Computational Analysis of miRNAs and Heterogeneous Genes associated with Vitiligo

Major Project Report

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Submitted by

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CERTIFICATE

This is to certify that this M. Tech. Major Project entitled ***"Computational Analysis of miRNAs and Heterogeneous Genes associated with Vitiligo"***, submitted by **Razia Rahman (DTU/MTECH/2K15/48)** in partial fulfillment of the requirements for the degree of Master of Technology in Biomedical Engineering, Delhi Technological University (Formerly Delhi College of Engineering), India, is an authentic record of the candidate's own work which has been carried out by her under my supervision.

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DECLARATION

I declare that my major project entitled ***"Computational Analysis of miRNAs and Heterogeneous Genes associated with Vitiligo"*** **submitted** to the Department of Biotechnology, Delhi Technological University, is a result of the work carried out by me at ***"Complex Systems and Genome Informatics Laboratory"***, Department of Biotechnology, as Major Project-II.

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Title: *"Computational Analysis of miRNAs and Heterogeneous Genes associated with Vitiligo"*

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ABSTRACT

Vitiligo is a polygenic disorder which results in the progressive loss of functional melanocytes and is characterized by the occurrence of depigmented patches on the skin. A comprehensive understanding of the molecular mechanisms that determine disease susceptibility, its onset, and phenotypic expression remains a challenge since the entire spectrum of this disorder is not yet clearly understood. Emerging evidence over the decades underlines the existing connection between deregulated miRNA function and disease pathogenesis. Unraveling the genetic variants that influence disease development may also provide key insights into the rationale for disease susceptibility. In the present work, we applied a systems biology approach to identify the role of potential miRNAs and susceptible gene variants associated with vitiligo. We further identified the miRNA target genes and constructed a miRNA-target gene network that revealed essential miRNAs that might be fundamentally linked to vitiligo. Our protein-protein interaction (PPI) network in combination with drug-target network highlighted potential protein targets which may be used as novel drug candidates. We also performed functional module and pathway analysis of the vitiligo associated proteins to explore their significance in disease onset. Furthermore, we investigated the pathogenic effect of the plausible single nucleotide polymorphisms (SNPs) that could affect protein structure and function and conducted preliminary protein modeling to implicate the role of SNP in disease pathogenesis. Thus, our analysis unveiled significant findings that may drive the way towards better therapeutic interventions for vitiligo management.

KEYWORDS: Vitiligo, miRNA, SNP, miRNA-target gene network, PPI network, drug-target network

CHAPTER 1

INTRODUCTION

Vitiligo is a chronic, acquired depigmentation disorder of the skin which results in an episodically progressive loss of functional melanocytes causing pigment dilution in the affected areas of the skin (Picardo et al., 2015). It is characterized by the appearance of asymptomatic, non-scaly white macules with sharply demarcated margins on the skin (Ezzedine et al., 2012). Since it involves the follicular melanocyte reservoir, it is also accompanied by whitening of the hair (Taïeb and Picardo, 2007; van Geel et al., 2012), although, the skin and hair are affected at different degrees depending on the disease duration. The course of the disease is unforeseeable with the depigmented patches stabilizing eventually with the duration of the disease.

Affecting 0.5%-1% of the world population (Howitz et al., 1977; Boisseau-Garsaud et al., 2000) and with a prevalence rate of 0.5%-2.5% in India (Handa and Kaur, 1999), vitiligo can develop at any age irrespective of the type of skin, gender, race or geographical location. Half of the population develops the disease before the age of 20 years, and nearly 70% to 80% develops it before the age of 30 years (Sehgal and Srivastava, 2007) inflicting significant psychological stress and exerting a pernicious influence on the quality of life in patients concerning self-esteem and social interactions.

The exact cause of disease onset is yet a topic of debate with a broad range of theories implicated in the etiology and pathogenesis of vitiligo including immune-mediated mechanisms (Spritz, 2007), increased oxidative stress (Jimbow et al., 2001; Yildirim et al., 2004), melanocyte growth factors and defective melanocyte adhesion (Gauthier et al., 2003), genetic susceptibility (Spritz 2012), viral infections (Akbayir et al., 2004; Akcan et al., 2006; Toker et al., 2007; Niamba et al., 2007), and neurogenic mechanisms (Al'Abadie et al., 1994). However, recent research has established the theory of autoimmune-mediated destruction of melanocytes as the current leading hypothesis. Therefore, the key to minimizing the impression of vitiligo lies in the better understanding and prospective manipulation of biological mechanisms entailing vitiligo etiopathogenesis.

Vitiligo appears to be a multifaceted disorder underlying both genetic and non–genetic (environmental) factors in a complex interactive manner. Recent advances in genetic studies have led to the considerable progress in defining the genetic epidemiology and pathogenesis of vitiligo, and its relationships to other autoimmune diseases offering a real insight into its biological framework leading to a more effective treatment approach as well as disease

prevention (Spritz, 2013). Almost all genetic studies have been implemented on generalized (non-segmental) vitiligo; hence, we considered generalized vitiligo for the present study.

MicroRNAs have been implicated to have significant roles in regulating various physiological and developmental processes in humans (Sayed and Abdellatif, 2011), including the development and function of melanocytes as well as immune cells (Mansuri et al., 2016). The identification of the miRNAs involved in vitiligo pathogenesis may serve as novel biomarkers for disease prognosis. Also, strong evidence for genetic factors in the pathogenesis of generalized vitiligo from various gene expression and association studies identified candidate genes, those encoding components of biological networks that primarily regulate the elements of the immune system and their targeted destruction of melanocytes mediating vitiligo susceptibility (Spritz, 2012). Genes tend to work together developing an intricate network of interactions. The complete sequencing of several genomes has uncovered that the primary provenance of the complexity of human disorders is in parallel with the genetic variations attributable to single nucleotide polymorphisms rather than barely protein-protein interactions. The interaction of genetic variants and the distribution of SNPs in the genome are firmly believed to contribute to the genetic risk of phenotype variation in human complex diseases (Cordell, 2009; Shen et al., 2016).

In the present work, we investigated the specific miRNAs reported to be associated with vitiligo and identified their target genes along with the susceptible genetic variants and their pathogenic effect using computational platforms. The cataloging of susceptible miRNAs and SNPs is essential for narrowing down the plausible concomitant genetic determinants of vitiligo. Protein-protein interactions are virtually intrinsic for every cellular and regulatory process, and a damaging alteration in such interactions have been deduced to cause and sometimes even accelerate human diseases. The regulation or impediment of a known detrimental protein-protein interaction delineates a principal target for drug discovery. Hence, a systems biology approach was implemented that unveiled significant interconnections and revealed intricate patterns of disease association. Such a network analysis is helpful in studying the gene expressions and analyzing a large set of disease-associated proteins. Together with gene ontology analysis, it contributes to deciphering the regulatory networks and pathways underlying cellular responses and elucidating the mechanisms that underpin complex diseases. Furthermore, we prioritized a few proteins in our protein-protein interaction network as pertinent hub proteins which may be targeted for treating vitiligo and scrutinized drug-target and drug-similarity interactions with an effort to provide potential novel targets for optimal therapeutic interventions. The present network integration approach attempts to furnish a comprehensive understanding of the biological mechanisms that mediate disease pathogenesis, offering novel interventional drug targets for both the treatment and prevention of vitiligo in genetically susceptible individuals.

CHAPTER 2

This chapter provides an overview of vitiligo and its types, the pathophysiology of the disease, therapeutic options, and management of disease, its association with other diseases, the role of miRNAs and genetic variants on disease susceptibility and the importance of interaction network studies and polypharmacological studies in disease modules.

REVIEW OF LITERATURE

2.1. Overview

Evolutionary studies indicated that skin pigmentation was the result of adaptive responses to the environment (UV radiation) in humans. Pigmentation provides photoprotection and participates in skin barrier function and antimicrobial defenses of the skin, hence, is essential for body homeostasis (Jablonski and Chaplin, 2010). Vitiligo is a complex depigmentation disorder leading to the selective disappearance of functional melanocytes affecting the life of patients both biologically and psychologically. It is characterized by white non-scaly patches with distinct sharp margins distributed unilaterally in the skin. The development of new lesions resulting in enlarged macules is classified as an active form of the disease (Gawkrodger et al., 2010). Figure 1 shows the major classifications of vitiligo, namely, non-segmental or generalized vitiligo, segmental vitiligo and mixed vitiligo. The onset of mixed vitiligo is the same as segmental vitiligo which eventually develops into non-segmental vitiligo; thus, the name mixed vitiligo.

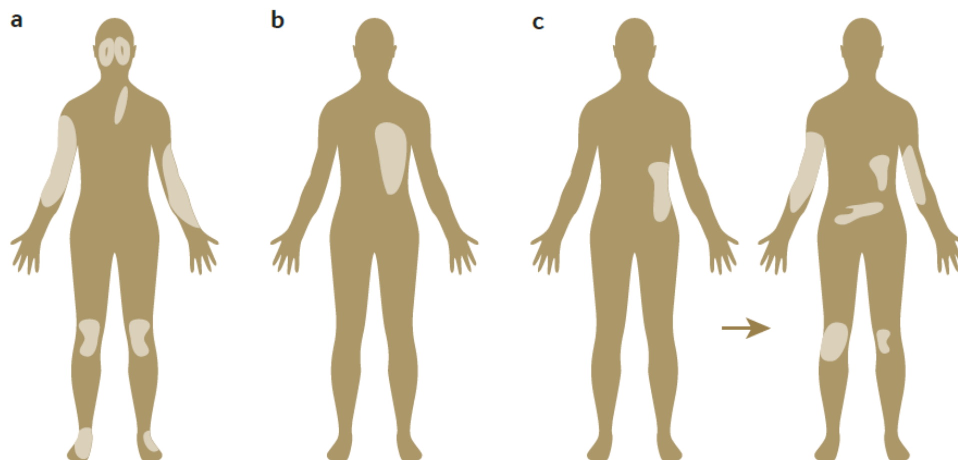

Figure 1: Types of vitiligo: a) non-segmental or generalized vitiligo, b) segmental vitiligo and c) mixed vitiligo (Picardo et al., 2015).

In non-segmental (generalized) vitiligo, which is the most common form of vitiligo, the depigmented patches develop on both sides of the body (Taı¨eb and Picardo, 2009). While, in segmental vitiligo, the patches are limited to only one side of the body, particularly in the face and trunk area and do not usually cross the midline of the body. In segmental vitiligo, typical distribution patterns on the face and trunk have been described, which aid in differential diagnosis (Kim et al., 2011; Geel et al., 2014). The markedly different distribution patterns aid in recognition of the type of vitiligo as the evolution and the kind of treatment is different for the different subtypes of vitiligo. Mixed vitiligo, on the other hand, has been described as a rare combination of both segmental vitiligo and nonsegmental vitiligo (Ezzedine et al., 2011).

2.2. Pathophysiology of vitiligo

Though the mechanisms leading to vitiligo is a still a debatable topic, however, several hypotheses have been presented signifying its association with the onset of vitiligo. Among the different theories developed, namely, oxidative stress, melanocyte growth and defective melanocyte adhesion, viral infections and neural mechanisms, the autoimmune theory is currently the leading theory.

2.2.1. Autoimmune theory

The loss of self-tolerance in the pathogenesis of vitiligo is unclear and not yet well understood. High levels of circulating melanocyte autoantibodies recognized by T cells have been found in many vitiligo patients with their role being linked to the destruction of keratinocytes and melanocytes (Le Poole and Luiten, 2008). Several studies have shown the accumulation of T helper (TH) and T cytotoxic (TC) cells kill melanocytes in the junction of the dermal and epidermal area of vitiligo lesion implying cell-mediated immune response activity (Oyarbide-Valencia et al., 2006). Certain MHC alleles have been suggested to be associated with vitiligo as a vital link between the disease etiology and the aberrant self-antigen presentation to the T cells (Spritz, 2012). Also, the fundamental role of regulatory T cells (Tregs) in the pathogenesis of vitiligo has been implicated in several reports with a reduction in their number in the blood of vitiligo patients (Ben Ahmed et al., 2012; Lili et al., 2012; Dwivedi et al., 2015). Also considered as a Th1-related disease, a significant increase in the concentration of the cytokines, namely, TNF-α, IFNG, IL-10 and IL-17 have also been reported to be associated with the onset as well as persistence of vitiligo in patients (Taher et al., 2009). Therefore, vitiligo serves as an eminent disease model to understand the initiation and progression of organ-specific autoimmune diseases.

2.2.2. Oxidative stress theory

Oxidative stress, which is a result of increase in the levels of reactive oxygen species (ROS) compromises the function of cellular proteins and membrane lipids, thus, impairing the activity

of the antioxidant system in both lesional and non-lesional skin (Maresca et al., 1997; Schallreuter et al., 2008). This imbalanced status of the antioxidant system in vitiligo has been indicated to cause increased sensitivity of melanocytes to oxidative stress leading to cellular death (Jimbow et al., 2001). Superoxide dismutase, an antioxidant enzyme, has been reported to be altered in vitiligo skin indicating that ROS generation causes an alteration in the expression of the antioxidant system affecting melanocyte function (Sravani et al., 2009). Oxidative stress-driven reduction of TRP1 expression triggers the production of intermediates of toxic melanin leading to subsequent immune-mediated melanocyte destruction (Dell'anna and Picardo, 2006).

2.2.3. Deficient melanocyte adhesion and melanocyte growth theory

Several evidence of the decreased adhesive property of melanocytes in vitiligo has been reported by various research groups (Gauthier et al., 2003). Reduced expression levels of E-cadherin have been observed in melanocytes prior to depigmentation development in vitiligo skin. During oxidative or mechanical stress, an altered E-cadherin expression incites loss of adhesion in epidermal melanocytes due to the increased levels of anti-adhesion molecule, tenascin (Le Poole et al., 1997; Wagner et al., 2015). Loss of melanocytes from the epidermal layer due to deficient adhesion of melanocytes could be an early phenomenon in vitiligo. Also, the factors influencing successful differentiation and proliferation of melanocytes, and their appropriate migration may also render susceptibility to vitiligo.

2.2.4. Viral theory

Several studies have depicted a strong association between vitiligo and hepatitis C virus (HCV) and hepatitis B virus (HBV) infections in vitiligo patients (Akbayir et al., 2004; Akcan et al., 2006). Also, the association of cytomegalovirus (CMV) infections with vitiligo was also suggested to provoke the deterioration of skin conditions in vitiligo (Toker et al., 2007). Furthermore, the suspicious association of herpes virus and the human immunodeficiency virus (HIV) infection with vitiligo has also been reported by research groups (Niamba et al., 2007).

2.2.5. Neuronal mechanisms theory

Clinical observations addressing the correlation of local neurological damage to skin depigmentation (whitening) suggests that neuronal mechanisms do have a role to play in vitiligo pathogenesis (Al'Abadie et al., 1994). Current evidence of the detection of neuropeptides in vitiligo lesions supports the neural hypothesis which might be the effect of inflammation rather than a triggering factor. An increased level of neuropeptides such as NPY has been observed in the marginal areas of vitiligo lesions triggered under the conditions of oxidative stress that is thought as a reason for the induction of disease (Lazarova et al., 2000).

2.3. Therapeutic options and management of vitiligo

The management of vitiligo becomes challenging considering its complex etiopathogenesis. Therefore, the current optimal management options of vitiligo according to the recent consensus guidelines involves a personalized approach with the therapeutic choice influenced by several factors such as disease course and its impact, skin type, age, gender, age, affected area and its extent, and social and cultural life influences (Taieb et al., 2013).

2.3.1. Topical corticosteroids, immunomodulators, and antioxidants

Topical corticosteroids being the first-line of treatment option manages disease progression by initiating anti-inflammatory responses with trivial outcomes. Although repigmentation is observed in the face and neck, the trunk area and the extremities show limited repigmentation. Oral corticosteroid involving moderate dosage of corticosteroids (mini-pulse therapy) is also used to arrest disease progression with rare repigmentation outcomes. However, the associated side effects limit its long-term use (Njoo et al., 1998).

Topical immunomodulators such as tacrolimus and pimecrolimus attenuate the production of proinflammatory cytokines by inhibiting T cell activity, thereby, enhancing melanocyte migration and pigmentation in vitiligo patients. Similar to corticosteroids, the results mostly show repigmentation in the face with moderate effects at other sites of the body (Ormerod, 2005).

Although, according to the current consensus guidelines, the use of topical antioxidants is not recommended, however, they are frequently prescribed in relatively limited trials (Leone and Paro, 2015). The use of oral antioxidants in combination therapy is sometimes considered in patients undergoing phototherapy.

2.3.2. Phototherapy

Narrow-band UVB (NB-UVB) phototherapy is an effective treatment choice that has long been recognized to induce repigmentation. The majority of the patients are observed to develop the signs of repigmentation with phototherapy. Topical treatments are also advised after completed phototherapy sessions to prevent recurrence of depigmentation (Sitek et al., 2007).

Photochemotherapy is also an option, but the recurrent side effects often accompany carcinogenic risk along with limited successful outcomes which restrict its use over NB-UVB where such risks are less evident (Bhatnagar et al., 2007)

2.3.3. Surgery

Pigment cell transplantation techniques such as cellular and tissue graft transplantation may offer a valuable alternative treatment option. The necessity of the disease stability, which is the primary criteria linked to successful outcomes, limits this treatment option to selective patients only (van Geel et al., 2010). Regardless of the technique used, the stability of the lesions is a

major criterion related to the outcome of the procedure. It is effective in both stable non-segmental and segmental vitiligo patients.

2.3.4. Combination Therapy

The complexity of the disease makes it necessary to use a combination of different treatments to address both inflammatory responses on melanocyte differentiation and proliferation. Currently, NB-UVB is prescribed along with topical corticosteroids and immunomodulators, instead of its use as a monotherapy. Such combination therapies have been shown to accelerate the repigmentation rates (Nordal et al., 2011). However, the risk of skin cancer due to combined use of immunosuppressants is a topic of concern and debate.

2.3.5. Depigmentation and cosmetic camouflage

In conditions of extensive vitiligo where most parts of the body are affected by depigmentation, depigmenting the remaining pigmented areas is considered as a better option instead of repigmentation treatments. Bleaching creams, laser therapy, and cryotherapy are some of the options for depigmentation therapy. Regardless of this treatment method, repigmentation on the treated depigmented areas might occur and as such a permanent cure cannot be assured (AlGhamdi and Kumar, 2011).

Camouflaging the depigmented areas with cosmetic products could aid to reduce the daily impact of the disease on social life. Specialized advice is required to be taken before going for such alternatives of cosmetic exposure to vitiligo skin (Hossain et al., 2016).

2.4. Association with other diseases

Due to the polygenic nature of vitiligo, it is frequently associated with several autoimmune or autoinflammatory diseases, namely, thyroid disorders (Kasumagic-Halilovic et al., 2011), psoriasis, atopic dermatitis (Ezzedine et al., 2011), diabetes mellitus, pernicious anemia and Addison’s disease (Rezaei et al., 2007). Antibodies directed against melanocytes and other organ-specific tissues have been found in vitiligo patients. Recent observations by genome research groups strongly point to vitiligo as an autoimmune disease sharing genes with other autoimmune disorders (Jin et al., 2012). Although the definite link between vitiligo and melanoma has not been fully elucidated yet, it has been reported that they both share an inverse relationship meaning that vitiligo affected people have a much lower risk of developing melanoma (Spritz, 2007).

2.5. Role of miRNAs and genetic variants on disease susceptibility

Various genetic and biological studies have improved our knowledge on vitiligo patho¬genesis and have opened up new avenues for novel targeted therapies for lack of melanocyte regeneration in vitiligo.

Recent studies have shown that miRNAs play prime roles in various cellular, regulatory and signaling processes maintaining physiological homeostasis. They are also essential for cellular morphogenesis, and any disruption in its architecture leads to disease development and progression. Deregulated miRNA metabolism is indicative of inflammatory skin conditions and linked to vitiligo pathogenesis (Mansuri et al., 2014). MicroRNAs are conserved, small, endogenous non-coding RNA molecules that regulate post-transcriptional gene expression. It promotes translational repression by targeting specific mRNAs based on their complementarity degree leading to the cleavage and degradation of mRNA. At present, characterization of interpretative miRNA expression and function in human melanocytes has elucidated them to be promising biomarkers for disease prognosis.

In the recent years, several genome-wide association and linkage studies have been conducted in vitiligo which is a polygenic disorder with a complex mechanism of pathogenesis. Accordingly, several potent disease contributing loci has been identified to be associated with vitiligo. The risk of developing vitiligo of the familial first-grade relatives signifies the heritability and genetic origin of vitiligo associated genes (Alkhateeb et al., 2003). Various studies and reports have identified that the majority of the susceptible gene variants inculpates modulation of the immune system along with genes linked with melanocytes proliferation and migration (Zhang and Xiang, 2014). It is the modulation and alteration exerted by specific genetic variations and their interactions that predispose inflammatory responses targeting melanocyte death owing to the onset of vitiligo.

2.6. Importance of interaction network studies and polypharmacological studies in disease modules

Network science and analysis offer unforeseen perspective prospects to understand and analyze the internal cellular organization and the interconnections between disease-related genes and functional proteins (Barabási and Oltvai, 2004). Protein-protein interaction networks composed of multiple nodes connected by edges accommodate better estimation of network statistics contributing to a comprehensive assistance in discerning biological mechanisms and pathways that dictate the manifestation of a disease cycle. This is particularly propitious when interpreting polygenic disorders having intricate patterns. Protein-protein interactions are of prime importance for various cellular and regulatory processes. Genetic variation alters or damages protein structure inciting disruption in protein-protein interactions constituting the pretext of disease development. These interaction networks usually consist of a few essential nodes (called hubs) that show maximum interaction to a large number of neighboring nodes. According to the phenomena of the centrality-lethality rule, the identification of such hub proteins and their inhibition may be lethal for the network. The magnitude of the change in structure caused by the removal of a node in a network determines the relative importance of the node in the network. Removal of such structurally critical nodes (hubs) in a network is

widely believed to reflect the significance of the network architecture to better ascertain the network functionality (He and Zhang, 2006). These hubs in a protein-protein interaction network may represent potential drug candidates. Since drug discovery and development is a complicated and expensive process, polypharmacology has emerged as the next paradigm of drug discovery. The transformation in the philosophy of current drug designing from one drug-one target to multiple targets of a single drug incorporates polypharmacological analysis that intends to discover the unknown targets for the existing drugs (Yıldırım et al., 2007). Polypharmacology based integrated systems biology approaches along with computational modeling, pharmacological and clinical studies are productive for identification of novel molecular determinants essential in drug discovery and development. It also aids in unraveling the understanding of the significant impact of a new drug on complex human diseases. A drug showing connections with multiple nodes (targets) in a network implies its high efficacious potential to control or inhibit the function of the particular target that is detrimental for regulatory pathways (Boran and Iyengar, 2010). The identification of such hub proteins in a disease network together with polypharmacological studies serves as an effective practical approach towards better therapeutic interventions.

CHAPTER 3

This chapter provides an overview of the dataset collection, data sources and software used, and a detailed explanation of the methods used in the analysis as well as the reasons behind using those methods.

METHODOLOGY

In the present work, we implemented a systems biology approach to explore the role of miRNAs and its target genes, and genetic variants on the onset and progression of vitiligo and analyzed the biological and molecular activities and signaling pathways of the proteins associated with vitiligo. Computational analysis for harmful SNPs was also carried out followed by polypharmacological studies to identify potential drug candidates. A graphical representation of the workflow is shown in Figure 2.

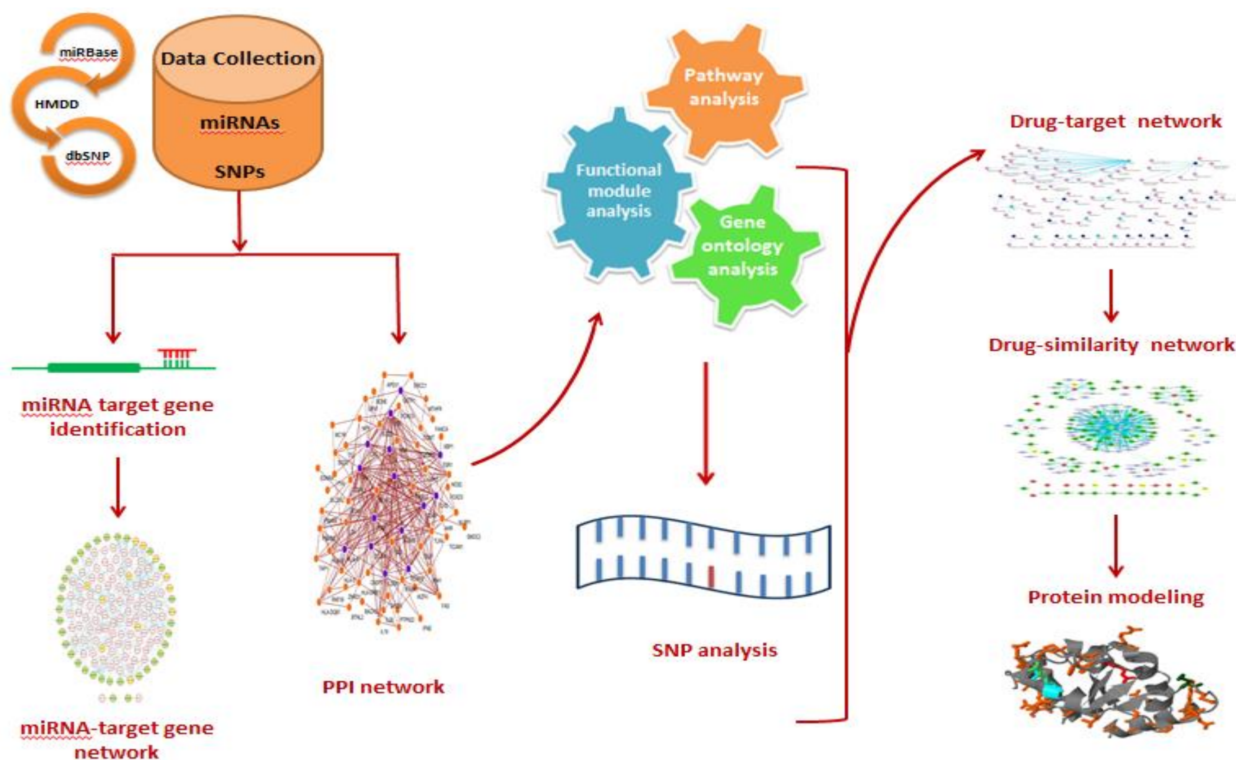

Figure 2: Graphical representation of the workflow.

3.1. Data Collection

To analyze the role of miRNAs associated with vitiligo, the miRNA information was obtained from literature search in NCBI PUBMED server (https://www.ncbi.nlm.nih.gov/pubmed/) and publicly available online databases, namely, HMDD v2.0 (the Human microRNA Disease

Database)(Li et al., 2014), Entrez GENE database of NCBI (https://www.ncbi.nlm.nih.gov/gene/), and miRBase (Griffiths-Jones, 2006). A total of 41 types of miRNAs were found to be associated with vitiligo. Information regarding the miRNAs was thus collected and used to develop a data sheet consisting information about the following attributes as under:

- miRNA: The name of the miRNA which is reported to be associated with the disease.
- PubMed ID (PMID): The reference numbers of the research articles in PubMed, from where the information of vitiligo associated miRNAs was retrieved.
- Expression: It represents the expression of miRNA in disease conditions whether it is upregulated or downregulated.
- p-value: It describes the significance of the results reported in research articles for a miRNA-disease association. We have considered p-value ≤ 0.05 to be significant for data collection.
- Chromosome: The chromosome number on which the miRNA gene is reported to be present in the Entrez Gene database of NCBI.
- Chromosome Location: The location of the miRNA gene in the chromosome as reported in the Entrez Gene database of NCBI.
- HGNC Symbol: The HUGO Gene Nomenclature Committee (HGNC) approved gene nomenclature for the corresponding miRNA gene as reported in miRBase.
- miRNA Sequence: The nucleotide sequence of the miRNA as reported in miRBase.

On the other hand, we extracted the information of genes associated with vitiligo and its SNPs from the GENE database and dbSNP of NCBI (https://www.ncbi.nlm.nih.gov/snp/). The build 141 of NCBI dbSNP database is the latest release containing nearly 44 million validated human SNPs (Sherry et al., 2001). A total of 186 genes were reported to be associated with the disease among which 134 polymorphisms for 84 genes were reported to be positively associated. UnitProt IDs (http://www.uniprot.org/) of the proteins were also noted. Information regarding the SNPs having significant p-value was thus collected and used to develop a data sheet consisting information about the following attributes as under:

- Gene Name: The name of the gene in which the SNP is reported to be associated with the disease.
- PubMed ID (PMID): The reference number of the research paper in PubMed, from where the information of vitiligo associated genes and SNPs were retrieved.
- rs ID: The accession number used to refer to specific SNPs as reported in dbSNP build 141.
- Odds Ratio (OR): It represents the likelihood of an outcome to occur in the presence of a given particular exposure. This field is left blank where the OR for a particular SNP is not reported in the particular research article.

- Population: The population which was studied in the particular research article.
- Geographical Location: The location, that is, the country name in which the study or research was reported to be conducted in the particular research article.
- Chromosome: The chromosome number on which the SNP is reported to be present in dbSNP build 141.
- Chromosome Position: The position of the SNP in the chromosome as reported in dbSNP build 141.
- Genotype: It represents the nucleotide change occurring due to polymorphism.
- Ancestral Allele: The wild-type allele which eventually changes into another due to polymorphism.
- Global MAF: The value of the second most frequent allele in a default global population as reported in dbSNP build 141 and is being provided to distinguish common variants from rare variants.
- p-value: It represents the significance of the results reported in research articles for a disease-SNP association. We have considered p-value ≤0.05 to be significant for data collection.
- Mutation/Variant: The type of SNP depending on whether it is found in the coding region or the non-coding region of the respective gene.
- SNP Location: The location of the SNP in the respective gene whether it is in the exon, intron, UTR or intergenic.
- Position: The position of the mutated amino acid in the protein. This field is left blank if the SNP is in the non-coding region of the corresponding gene.
- Reference contig: The NT accession numbers of the reference contig in which the SNP was reported in dbSNP build 141.
- Reference mRNA: The NM accession numbers of the reference transcript in which the SNP was reported in dbSNP build 141. This field is left blank if the SNP is not found in the transcript.
- Reference protein: The NP accession numbers of the reference protein sequence in which the SNP was reported in dbSNP build 141. This field is left blank if the SNP is not found in the transcript.
- Residue Change: The amino acid change occurring due to polymorphism.
- UniProt ID: The accession number of the protein as reported in UniProt database in which the SNP was found. This field is left blank if the SNP is not found in the transcript.

3.2. Identification of miRNA target genes

The miRNAs identified to be associated with vitiligo were used to find their respective target genes. TargetScanHuman 7.1 (Agarwal et al., 2015), which is free online web tool, was used to

detect targets in the 3'UTR of the protein-coding transcripts by base-pairing rules where predictions with both broadly conserved and poorly conserved sites are provided.

These target sites are the conserved sites that match or are complementary to the seed region of the miRNA that ultimately facilitates the binding of miRNA with the mRNA to functionally degrade the mRNA thereby resulting in gene silencing.

Information regarding the miRNA target genes was thus collected and used to develop a data sheet consisting information about the following attributes as under:

- miRNA: The name of the miRNA which is reported to be associated with the disease.
- Target Gene: The name of the gene which has the conserved 8mer or 7mer or 6mer target sites matching the seed region of the respective miRNA for miRNA-mRNA association to occur as reported in TargetScanHuman 7.1.
- Target Gene Name: The full name of the gene which is reported to be the target gene of the respective miRNA reported in TargetScanHuman 7.1.
- Representative Transcript ID: The transcript ID of the target gene as reported in Ensembl.

3.3. Construction of miRNA-target gene network

A structured network layout explaining network integrity is the core requirement to justify the interaction between miRNA and disease. A miRNA-target gene interaction network was constructed and analyzed to understand the miRNA-target gene relationship and validate the miRNA-disease association. The miRNA-target gene bipartite network consists of two sets of nodes-one set represents the miRNAs, and the other set represents the target genes. Nodes from the two sets were connected if a particular miRNA is associated with a particular target gene. The datasheet prepared which included all the miRNAs associated with vitiligo along with their target genes was used to generate the network in Cytoscape (Shannon et al., 2003).

Cytoscape, which is a software package, was used for modeling, visualizing, and analyzing genetic and molecular interaction network. It offers a variety of plugins and applications relating to the different aspects of systems biology.

Cytoscape's software probes the network and provides a basic functionality layout to visually assimilate and understand the network with phenotypes, expression profiles, relevant molecular states, and targets. This results in a swift development of supplementary computational analysis and topographies linking the network to functional annotations databases. After obtaining a miRNA-disease bipartite network, the hub miRNA analysis for the identification of essential miRNAs was performed.

3.4. Construction of vitiligo associated protein-protein interaction network

Interactions between genes whose expression profiles are correlated with disease pathogenesis may contribute to the progression of the disease. To identify such interactions, we constructed the protein-protein interaction network using STRING (The Search Tool for the Retrieval of Interacting Genes) (Von Mering et al., 2005).

It is a comprehensive database capable of providing an overall view of all the known and predicted protein-protein interactions of physical and functional associations. The data compiled in the database emerges from various computational predictions, conserved co-expressions of genes, experimental studies, and from interactions aggregated from other (primary) databases. It consists of approximately 9.6 million proteins in over more than 2000 organisms. The PPI network generated based on STRING online database was then visualized in Cytoscape whose common feature lies in combing biological interaction networks with relevant large databases into a unified framework.

3.5. Functional module and enrichment analysis

Considering the connectivity properties of a network, we resolved to identify the significant clusters or modules enriched in biological processes from the complex bipartite network to extract biologically meaningful interactions. The modular analysis can provide a better insight into the relationship of the interconnected proteins assuming that the highly connected nodes in a network could form a cluster. As we know, cellular processes and functions are modular; therefore, it is more feasible to predict the structural and functional behavior of a particular module than that of an individual gene. Such module analysis has played a significant role in the past in determining disease mechanisms (Mitra et al., 2013).

We used Markov Clustering Algorithm (MCL) method among the other clustering algorithms provided in Cytoscape as it is the most commonly used unsupervised clustering algorithms for functional module analysis and assigns a fast and reliable scalable method for finding functionally enriched clusters in complex networks. The granularity parameter for MCL clustering was kept at 1.8.

To measure the individual relationship of the genes based on the degrees of their co-association with diseases, we further performed functional enrichment analysis of the clustered groups of genes in the network using DAVID (The Database for Annotation, Visualization and Integrated Discovery) database 6.8 (Huang et al., 2009), to interpret their biological impetus. Functional enrichment analysis, also known as gene enrichment analysis is a method which uses statistical approaches to identify the clusters or classes of genes or proteins that are highly expressed or enriched in a large set of genes or proteins and these clusters may have an

association with disease phenotypes (Huang et al., 2008). For researchers performing high-throughput experiments that generate large sets of genes, for example, genes that are differentially expressed under different conditions, it becomes necessary for them to retrieve a functional profile of that gene set to generate a better understand the underlying biological processes.

All the genes were mapped into the DAVID which is an online free database of web-accessible programs that delivers a comprehensive set of functional annotation tools for researchers to evaluate the biological meaning behind large list of genes. The default settings with 'Homo sapiens' was selected as the species background as well as the current background for the analysis.

3.6. Ontology analysis

Gene ontology analysis describes gene products in context to their association in the biological processes (pathways and greater routes made up of the activities of numerous gene products), cellular components (functional site of the gene products) and molecular functions (molecular activities of associated gene products) in a species-independent manner. The gene ontology outlines concepts or classes which are used to define gene function and the relationships between these concepts considering that a significant fraction of the genes specific for the core biological functions is shared by all eukaryotes (Mi et al., 2017). This information about the shared genes and proteins facilitates and contributes to our apprehension of all the diverse organisms that share them.

Central Gene Ontology Consortium server is a widely accepted source of functional gene annotation, collaborating many databases that facilitate uniform queries across all of them. Thus, it was used to describe and analyze the molecular functions and biological processes and pathways for the selected target gene set of interest in which they were significantly involved. For gene ontology analysis, the default settings with 'Homo sapiens' was selected as the species background as well as the current background. Also, PANTHER (Protein Analysis Through Evolutionary Relationship) (Mi et al., 2013), classification system which also offers spontaneous visualization of images of GO analysis was used. It categorized the vitiligo associated genes simplifying a high throughput analysis for better understanding of the biological processes and molecular functions relationships in which these genes were involved.

3.7. Pathway analysis

To analyze the pathways in which these genes are involved, we conducted a pathway analysis using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways in DAVID 6.8. KEGG Pathway database is the most comprehensive and widely used database of annotation information. The

pathway classification with p≤0.001 and Benjamini-Hochberg FDR ≤0.01 was considered to have the most biological significance. Pathway analysis helps to interpret the data in the context of biological interactions and identify related proteins within a pathway.

A single protein may be involved in multiple pathways that are of importance to many biological processes. The biological cause of a disease can be explored by examining the changes in gene expression in a pathway. Also, the same pathway can be targeted for novel drug candidates (Wang et al., 2010). Deciphering the pathways which are explicitly targeted by the essential proteins may provide insight into their regulatory mechanisms. To better analyze the pathway analysis results, we constructed a protein pathway network to elucidate which pathways were eminently targeted by the proteins, specifically, the hub proteins. Such pathways, in turn, can be targeted for therapy and treatment approaches.

3.8. SNP analysis

The feasibility of the identification of the nsSNPs that vest susceptibility or resistance to human diseases has been improved with the use of in silico tools. To elucidate of the function of mutations in vitiligo susceptible genes, we investigated the pathogenic effect of 134 SNPs which were reported to be associated with vitiligo. Among the 134 SNPs, the functional context of 36 nsSNPs was analyzed by employing various computational platforms. We used a combination of computational tools, namely, SIFT (Kumar et al., 2009), PolyPhen 2.0 (Adzhubei et al., 2010), PROVEAN (Choi et al., 2012), SNPs&GO (Magesh and Doss, 2014), I-Mutant Suite 3.0 (Capriotti et al., 2008), and PANTHER Evolutionary Analysis of Coding SNP (Mi et al., 2005) to identify the nsSNPs that potentially affect the structure and function of proteins associated with vitiligo.

- SIFT (Sorting Intolerant from Tolerant): A sequence-homology-based tool to predict whether an amino acid substitution would be tolerated or damaging in a protein (Kumar et al., 2009). The SNP IDs were queried and the tool considers SNPs as deleterious variants whose tolerance index score is ≤0.05. Available from: (http://sift.bii.a-star.edu.sg/index.html).
- PolyPhen 2.0 (Polymorphism Phenotyping v2): A software tool that predicts the likely impact of an amino acid substitution on the structure and function of a protein by comparing the structural and evolutionary information (Adzhubei et al., 2010). The protein sequence along with their amino acid substitution is queried to generate an output score ranging from 0 to 1. A zero indicates a neutral effect, whereas, a high score represents a variant that has a damaging effect of amino acid substitutions on the protein. Available from: (http://genetics.bwh.harvard.edu/pph2/).
- PROVEAN (Protein Variation Effect Analyzer): A software tool that predicts the functional impact of nsSNPs in a protein sequence (Choi et al., 2012). PROVEAN

prediction analysis in based on a cutoff score (=0.05) which indicates a deleterious substitution. Variants with a score above the threshold are considered to be neutral (Manickam et al., 2014). Available from: (http://provean.jcvi.org/index.php).

- SNPs&GO: A web server that predicts the probability of a protein variation to be significantly associated with a disease (Magesh and Doss, 2014). The queried protein sequence along with the mutational substitution and position yields a probability score. A score above 0.5 is considered to be deleterious. Available from: (http://snps.biofold.org/snps-and-go/snps-and-go.html).
- I-Mutant Suite 3.0: A support vector machine (SVM)-based prediction tool to predict the change in protein stability according to change in free energy, enthalpy, heat capacity, and temperature (Capriotti et al., 2008). The protein sequence along with the substitution position and the correlated new residue was queried for the analysis. The output result predicts the free energy change (DDG) and classifies it into any three of the mentioned classes, namely, largely unstable (DDG < −0.5 kcal/mol), largely stable (DDG>0.5 kcal/mol), or neutral (-0.5≤ DDG≤0.5 kcal/mol). The output result of a protein as largely unstable indicates the effect of a deleterious nsSNP. Available from: (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi).
- PANTHER Evolutionary Analysis of Coding SNP: A comprehensive system that evaluates the likelihood of a particular nsSNP to cause an impact on the function and activity of protein. The prediction is based on a method called PANTHER-PSEP (position-specific evolutionary preservation) that calculates the preservation time, that is, how long a given amino acid substitution has been conserved in the lineage (Mi et al., 2005). A longer preservation time implies a greater possibility of functional impact on a protein. Available from: (http://pantherdb.org/tools/csnpScoreForm.jsp).

Additionally, MutPred 2 (http://mutpred.mutdb.org/) (Pejaver et al., 2017) was used to interpret the possible molecular cause of disease-inducing amino acid substitutions. Furthermore, NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/) (Petersen et al., 2009) was used to analyze the effect of such mutations in the stability of the protein by predicting the solvent accessibility of the substituted residue. Since UTRs have an extensive role in the post-transcriptional regulation of gene expression which may affect the degradation or translational suppression of mRNA, UTRScan (Pesole et al., 1999) was carried out for the 24 UTR SNPs and PolymiRTS (Bhattacharya et al., 2013) was used to predict the effect of 3' UTR SNPs in regulatory mechanisms

- UTRScan: A program that identifies functional SNPs in the 5’ and 3’ UTRs. It searches the user submitted query FASTA sequence and looks for patterns defined in the UTRsite database which is a collection of experimentally determined regulatory elements located in the 5’ and 3’UTRs. A particular UTR SNP is predicted to have functional

significance if a mutational substitution results in a change in the functional patterns in the UTR. Available from: http://itbtools.ba.itb.cnr.it/utrscan.

- PolymiRTS: A database specifically designed for the analysis of non-coding SNPs, namely 3′ UTR. It predicts the variations in microRNA seed regions and target sites providing a more complete and accurate output. The results generated are assigned into four classes: 'D' (the SNP disrupts a conserved microRNA site); 'N' (the SNP disrupts a non-conserved microRNA site), 'C' (the SNP creates a new microRNA site); and 'O' (other cases when the ancestral allele cannot be determined). The class 'C' may cause abnormal gene repression whereas the class 'D' may cause the loss of normal repression control (Bhattacharya et al., 2013). Hence, they are most likely to have a functional impact. Available from: http://compbio.uthsc.edu/miRSNP/.

The information from different computational platforms was combined to prioritize the deleterious SNPs to increase the predictive power and accuracy of the results of the in silico techniques.

3.9. Construction of drug-target network

A single drug can target multiple proteins. To analyze this relationship between drug and protein targets (disease-gene products) and to understand how they intervene therapeutically in disease processes, we constructed a drug-target network (Yıldırım et al., 2007). We extracted information about drugs with respect to our drug candidates from DrugBank (Knox et al., 2011), which is a chemoinformatics resource that is updated and maintained with The Food and Drug Administration (FDA) information.

DrugBank combines extensive information on the chemical, pharmacological and pharmaceutical nature of drugs with comprehensive drug-target information about their mechanisms and targets. Currently, this database covers 8261 drug entries including 2254 FDA-approved drugs, 2021 FDA-approved small molecule drugs, 336 FDA-approved biotech (protein/peptide) drugs, 94 nutraceuticals and over 5019 experimental drugs. It supports a broad range of commercial drug names and its corresponding chemical composition. It even includes drugs that are at their investigational or experimental stages and specifically mentions the drugs that are illicit or withdrawn. It also provides information on drug metabolism, absorption, distribution, metabolism, excretion, and toxicity (ADMET) and other categories of quantitative structure-activity relationships (QSAR) with a purpose to encourage research in pharmacokinetics, pharmacodynamics, and drug design and discovery.

We used known FDA drugs (approved and approved-investigational) with respect to the protein targets to generate the drug-target bipartite network.

3.10. Construction of drug-similarity network

To identify which drugs act similarly on the same target, we constructed a drug-similarity network to analyze the interactions of drugs and its action on protein targets. DrugBank provides clinically relevant drug interactions, and the information of the interacting drugs and its interacting mechanism with respect to a particular drug was taken from DrugBank. Such a network can provide insights on drug-drug interaction for a potential drug candidate where an interacting drug can act synergistically or antagonistically with another drug altering the benefit or effectiveness of the drug on disease conditions. An interacting drug of a particular drug can also pose as a potential drug for the protein targeted by that drug.

3.11. Protein modeling of TLR2

Finding the 3D structure of proteins is helpful in predicting the impact of SNPs on the structural level and in showing the degrees of alteration. To elucidate the molecular dynamic behavior of the SNP in TLR2 protein, we performed preliminary protein modeling by G23D (Genomic variant to 3D protein data) (http://www.sheba-cancer.org.il/G23D) (Solomon et al., 2016) which is a tool for the conversion of human genomic coordinates to protein structures.

G23D allows the mapping of evolutionary related as well as identical protein of genomic variants in a 3D model structure assisting in the feasibility of structural insight. Along with the mutated sites, it also displays the wild type residue and other functional sites on the modeled 3D protein structure to facilitate better interpretation of the variant. The UniProt ID of the protein is submitted as the query along with the substitution position and the residual change. The genomic coordinates are converted to protein coordinates (models) using dbNSFP which are retrieved from ModBase, which is a database of theoretical models. BLAST search is then carried out for the input protein sequence with the amino acid substitution against the structures available in PDB. The sequence position is converted to structure coordinates within PDB files using S2C, a database correlating sequence with atomic residue and the side chains are modeled according to the mutation. The modeled 3D structure of the protein with its wild type and mutant residue is visualized in JSmol by the link generated in the results (Solomon et al., 2016).

We further carried out TLR2 protein-protein interaction analysis to analyze its interaction with other proteins which might be influenced by the mutation in TLR2 protein.

CHAPTER 4

This chapter provides an overview of the results obtained in the present work.

RESULTS

4.1. Data Collection

The datasheet comprising of 41 entries which include the collection of miRNAs associated with vitiligo is given in Figure 3. All the miRNA genes were present in the locus of almost all the chromosomes except for chromosome 9, 10, 13, 18, X and Y.

miRNA	PMID	Expression	p-value	Chromosome	Chromosome Location	Symbol	miRNA Sequence
hsa-miR-1	27450903	Up-regulated	<0.009	20	20q13.33	HGNC:MIR1	UGGGAAACAUACUUCUUUAUAUGCCCAUAUGGACCUGCUAAGCUAUGGAAUGUAAAGAAGUAUGUAUCUCA
hsa-miR-133b	17604727	Up-regulated	<0.035	6	6p12.2	HGNC:MIR133	CCUCAGAAGAAAGAUGCCCCCUGCUCUGGCUGGUCAAACGGAACCAAGUCCGUCUUCCUGAGAGGUUUGGUCC
hsa-miR-135a	27450903	Up-regulated	<0.0001	3	3p21.2	HGNC:MIR135A	AGGCCUCGCUGUUCUCUAUGGCUUUUUAUUCCUAUGUGAUUCUACUGCUCACUCAUAUAGGGAUUGGAGCCGU
hsa-miR-183	12554860	Up-regulated	<0.007	7	7q32.2	HGNC:MIR183	CCGCAGAGUGUGACUCCUGUUCUGUGUAUGGCACUGGUAGAAUUCACUGUGAACAGUCUCAGUCAGUGAAUU
hsa-miR-190	12554859	Up-regulated	<0.04	15	15q22.2	HGNC:MIR190	UGCAGGCCUCUGUGUGAUAUGUUUGAUAUAUUAGGUUGUUAUUUAAUCCAACUAUAUAUCAAACAUAUUCCU
hsa-miR-214	27450903	Up-regulated	<0.022	1	1q24.3	HGNC:MIR214	GGCCUGGCUGGACAGAGUUGUCAUGUGUCUGCCUGUCUACACUUGCUGUGCAGAACAUCCGCUCACCUGUACA
hsa-miR-301b	16954537	Up-regulated	<0.027	22	22q11.21	HGNC:MIR301B	GCCGCAGGUGCUCUGACGAGGUUGCACUACUGUGCUCUGAGAAGCAGUGCAAUGAUAUUGUCAAAGCAUCUG
hsa-miR-30a-3p	11679670	Up-regulated	<0.008	6	6q13	HGNC:MIR30A	CUUUCAGUCGGAUGUUUGCAGC
hsa-miR-375	17604727	Up-regulated	<0.023	2	2q35	HGNC:MIR375	CCCCGCGACGAGCCCCUCGCACAAACCGGACCUGAGCGUUUUGUUCGUUCGGCUCGCGUGAGGC
hsa-miR-487a	16274478	Up-regulated	<0.013	14	14q32.31	HGNC:MIR487A	GGUACUUGAAGAGUGGUUAUCCCUGCUGUGUUCGCUUAAUUUAUGACGAAUCAUACAGGGACAUCCAGUUUU
hsa-miR-517c	15965474	Up-regulated	<0.01	19	19q13.42	HGNC:MIR517C	GAAGAUCUCAGGCAGUGACCCUCUAGAUGGAAGCACUGUCUGUUGUCUAAGAAAAGAUCGUGCAUCCUUUUA
hsa-miR-616	27450903	Up-regulated	<0.023	12	12q13.3	HGNC:MIR616	UUAGGUAAUUCCUCCACUCAAAACCCUUCAGUGACUUCCAUGACAUGAAAUAGGAAGUCAUUGGAGGGUUUG
hsa-miR-211	21109473	Down-regulated	<0.014	15	15q13.3	HGNC:MIR617	UCACCUGGCCAUGUGACUUGUGGGCUUCCCUUUGUCAUCCUUCGCCUAGGGCUCUGAGCAGGGCAGGGACAGC
hsa-miR-136	15183728	Down-regulated	<0.04	14	14q32.2	HGNC:MIR618	UGAGCCCUCGGAGGACUCCAUUUGUUUUGAUGAUGGAUUCUUAUGCUCCAUCAUCGUCUCAAAUGAGUCUUCA
hsa-miR-296	12919684	Down-regulated	<0.023	20	20q13.32	HGNC:MIR619	AGGACCCUUCCAGAGGGCCCCCCCUCAAUCCUGUUGUGCCUAAUUCAGAGGGUUGGGUGGAGGCUCUCCUGAA
hsa-miR-328	27450903	Down-regulated	<0.009	16	16q22.1	HGNC:MIR620	UGGAGUGGGGGGGCAGGAGGGGCUCAGGGAGAAAGUGCAUACAGCCCCUGGCCCUCUCUGCCCUUCCGUCCCC
hsa-let-7c	11679670	Up-regulated	<0.039	21	21q21.1	HGNC:MIR621	GCAUCCGGGUUGAGGUAGUAGGUUGUAUGGUUUAGAGUUACACCCUGGGAGUUAACUGUACAACCUUCUAGC
hsa-miR-100	11914277	Up-regulated	<0.005	11	11q24.1	HGNC:MIR622	CCUGUUGCCACAAACCCGUAGAUCCGAACUUGUGGUAUUAGUCCGCACAAGCUUGUAUCUAUAGGUAUGUGUC
hsa-miR-10a	12624257	Up-regulated	<0.043	17	17q21.32	HGNC:MIR623	GAUCUGUCUGUCUUCUGUAUAUACCCUGUAGAUCCGAAUUUGUGUAAGGAAUUUUGUGGUCACAAAUUCGUA
hsa-miR-1180	23034410	Up-regulated	<0.022	17	17p11.2	HGNC:MIR624	GCUGCUGGACCCACCCGGCCGGGAAUAGUGCUCCUGGUUGUUUCCGGCUCGCGUGGGUGUGUCGGCGGC
hsa-miR-135b	14691248	Up-regulated	<0.04	1	1q32.1	HGNC:MIR625	CACUCUGCUGUGGCCUAUGGCUUUUCAUUCCUAUGUGAUUGCUGUCCCAAACUCAUGUAGGGCUAAAAGCCAU
hsa-miR-145	26941046	Down-regulated	<0.012	5	5q32	HGNC:MIR626	CACCUUGUCCUCACGGUCCAGUUUUCCCAGGAAUCCCUUAGAUGCUAAGAUGGGGAUUCCUGGAAAUACUGUU
hsa-miR-155	26941046	Up-regulated	<0.014	15	15q25.1	HGNC:MIR627	CCAGUCACGUCCCCUUAUCACUUUUCCAGCCCAGCUUUGUGACUGUAAGUGUUGGACGGAGAACUGAUAAGGG
hsa-miR-194	12554859	Up-regulated	<0.043	1	1q41	HGNC:MIR628	AUGGUGUUAUCAAGUGUAACAGCAACUCCAUGUGGACUGUGUACCAAUUUCCAGUGGAGAUGCUGUUACUUU

Figure 3: Datasheet of miRNAs.

A total of 134 polymorphisms for 84 genes were found to be associated with vitiligo. Among the 134 SNPs, 36 are nsSNPs, 7 are coding synonymous SNPs, and 68 SNPs were found in the non-coding region.

All the SNPs were found to be at the locus of almost all the chromosomes except for chromosome 13, 18, 20 and Y. The datasheet consisting of 134 SNPs associated with vitiligo is given in Figure 4.

Gene Name	PubMed ID	rs ID	Odds Ratio (OR)	Population	Geographical Location	Chromosome	Chromosome Position	Genotype	Ancestral Allele	Global MAF	p-value	Mutation/Variant	SNP Location	Position	Ref_contig	Ref_mRNA	Ref_protein	Residue Change	UniProt ID
HLA-B	21951294	rs946892	0.77	Chinese Han	China	6	31291060	[A/G]	G	A=0.4700/235	<0.003		Intergenic		NT_007592.16				
HLA-A	25645285	rs9261394	1.6	Japanese	Japan	6	30096785	[A/G]	A	A=0.3724/1865	<0.0004		Intergenic		NT_007592.16				
HCG9	25645285	rs382337	1.67	Japanese	Japan	6	29976381	[C/T]	T	C=0.2993/1499	<0.0005	intron variant	Intron		NT_007592.16				
HLA-A	25645285	rs934883	2.31	Japanese	Japan	6	29896720	[C/T]	C	T=0.2508/1256	<0.05		Intergenic		NT_007592.16				
HLA-A	25645285	rs6457110	2.31	Japanese	Japan	6	29966104	[A/T]	A	A=0.3846/1926	<0.05		Intergenic		NT_007592.16				
ZNRD1	20410501	rs936675	1.65	Japanese	Japan	6	30056900	[C/T]	C	T=0.2192/1098	<0.0008	intron variant	Intron		NT_007592.16				
RNF39	20410501	rs690925	1.57	Japanese	Japan	6	30087866	[A/G/T]	T	G=0.4103/2055	<0.0001		Intergenic		NT_007592.16				
TNF	26125752	rs1800629	1.56	Middle Eastern		6	31575254	[A/G]	G	A=0.0903/452	<0.0003	upstream variant	near gene		NT_007592.16	NM_000594.3			
HLA-DRB1	26787888	rs9271597	1.77	European-de	Various	6	32623514	[A/T]	T	A=0.3884/1945	<0.05		Intergenic		NT_007592.16				
HLA-DRB1	26787888	rs9271600	1.77	European-de	Various	6	32623555	[G/T]	T	G=0.3884/1945	<0.05		Intergenic		NT_007592.16				
HLA-DRB1	26787888	rs9271601	1.77	European-de	Various	6	32623560	[A/T]	A	T=0.3884/1945	<0.05		Intergenic		NT_007592.16				
HLA-C	22125590	rs946892	0.75	Chinese Han	China	6	31291060	[A/G]	G	A=0.4700/235	<0.01		Intergenic		NT_007592.16				
HERC2	22561518	rs1129038	1.22	European-de	United States	15	28111713	[A/G]	G	T=0.1769/886	<0.003	utr variant 3 prime	3-UTR		NT_010194.18	NM_004667.5			
HERC2	22561518	rs129138	1.22	European-de	United States	15	28120472	[A/G]	A	G=0.1773/888	<0.003	intron variant	Intron		NT_010194.18	NM_004667.5.			
FANCA	22561518	rs992629	0.79	European-de	United States	16	89751681	[A/G]	A	A=0.3339/1672	<0.0001	intron variant	Intron		NT_010498.16	NM_000135.2.			
TYR	22561518	rs440978	1.34	European-de	United States	11	95578258	[C/T]	T	C=0.1250/626	<0.0001		Intergenic		NT_033899.9				
IFIH1	22561518	rs2111485	0.77	European-de	United States	2	162254026	[A/G]	A	G=0.3393/1699	<0.005		Intergenic		NT_005403.18				
CD44	22561518	rs5937441	1.34	European-de	United States	3	119569567	[A/C]	A	C=0.2252/1128	<0.0003		Intergenic		NT_005612.17				
CLNK	22561518	rs1687257	1.21	European-de	United States	4	10725229	[C/T]	C	T=0.3960/1983	<0.0001	intron variant	Intron		NT_006316.17				
BACH2	22561518	rs375724	1.2	European-de	United States	6	90247744	[A/G]	A	T=0.3958/1982	<0.0002	intron variant	Intron		NT_025741.16	NM_021813.3.			
TG	22561518	rs853308	1.2	European-de	United States	8	132917672	[A/G]	A	T=0.3526/1766	<0.0001	intron variant	Intron		NT_008046.17	NM_003235.4.			
CASP7	22561518	rs3814231	0.81	European-de	United States	10	113721259	[A/G]	G	T=0.2612/1308	<0.0003	intron variant	Intron		NT_030059.14	NM_001227.4.			
SLC1A2	22561518	rs107681	1.21	European-de	United States	11	35259305	[A/G]	A	G=0.3602/1804	<0.0001	utr variant 3 prime	3-UTR		NT_009237.19	NM_001195728.2			
IKZF4	22561518	rs245698	1.29	European-de	United States	16	31963274	[C/T]	C		<0.0002		Intergenic		NT_187260.1				
SH2B3	22561518	rs3184504	0.76	European-de	United States	12	111446804	[C/T]	C	T=0.1474/738	<0.0002	missense	Exon	262	NT_029419.13	NM_005475.	NP_005466.	W [Trp] ⇒ R [Arg]	Q9UQQ2
ATX2	22561518	rs476657	0.76	European-de	United States	12	111466567	[A/T]	A	T=0.1512/757	<0.0003	intron variant	Intron		NT_029419.13	NM_002973.3.			
CD44	22561518	rs482202	0.78	European-de	United States	22	41361643	[A/G]	G	A=0.2486/1245	<0.0006		Intergenic		NT_011520.13				
TICAM1	22561518	rs6510827	1.19	European-de	United States	19	4830616	[C/T]	T	T=0.3251/1628	<0.0008	intron variant	Intron		NT_011295.12	NM_182919.3.			
PTPN22	18200060	rs2476601	2.16	Caucasian	United States	1	113834946	[A/G]	G	A=0.0274/137	<0.05	missense	Exon	620	NT_032977.10	NM_0011934	NP_0011803	R [Arg] ⇒ W [Trp]	Q9Y2R2
ATX2	21326295	rs7758128		European	United States	6	32377506	[A/C]	C	A=0.0473/237	<0.0005	intron variant	Intron		NT_007592.16				
BTNL2	21326295	rs28362680		European	United States	6	32403039	[C/T]	T	A=0.1887/945	<0.0001	missense	Exon	202	NT_007592.16	NM_0013045	NP_0012914	A [Ala] ⇒ V [Val]	Q9UIR0
BTNL2	21326295	rs28362683		European	United States	6	32405186	[C/T]	C	A=0.1344/673	<0.0001	synonymous codo	Exon	60	NT_007592.16	NM_0013045	NP_0012914	H [His] ⇒ H [His]	Q9UIR0
BTNL2	21326295	rs10947262		European	United States	6	32405535	[C/T]	T	T=0.1887/945	<0.0001	intron variant	Intron		NT_007592.16	NM_001304561.1.			
BTNL2	21326295	rs532098		European	United States	6	32610275	[C/T]	C	A=0.4269/2138	<0.0002		Intergenic		NT_007592.16				
IL2RA	20410501	rs1220649	1.58	European-de	United States	6	29969350	[A/G]	A	G=0.2604/1304	<0.05		Intergenic		NT_007592.16				
HCG9	20410501	rs382335	1.57	European-de	United States	6	29974306	[A/C/T]	C	T=0.2566/1285	<0.002	upstream variant	near gene		NT_007592.16				
HCG9	20410501	rs690402	1.56	European-de	United States	6	29975290	[A/G]	G	A=0.2560/1282	<0.0007	intron variant	Intron		NT_007592.16				
HCG9	20410501	rs532098	1.74	European-de	United States	6	32610275	[C/T]	C	A=0.4269/2138	<0.004		Intergenic		NT_007592.16				
BTNL2	20410501	rs3806156	1.53	European-de	United States	6	32405921	[G/T]	G	T=0.3966/1986	<0.004	intron variant	Intron		NT_007592.16	NM_001304561.1			
LPP	20410501	rs130763	1.3	European-de	United States	3	188371466	[C/T]	C	C=0.4904/2450	<0.003	intron variant	Intron		NT_005612.17	NM_005578.4			
LPP	20410501	rs1464510	1.32	European-de	United States	3	188394766	[A/G/T]	G	A=0.4427/2217	<0.006	intron variant	Intron		NT_005612.17	NM_005578.4			
IL2RA	20410501	rs706779	1.35	European-de	United States	10	6056861	[A/G]	A	C=0.4581/2294	<0.0001	intron variant	Intron		NT_008705.17	NM_000417.2			

Figure 4: Datasheet of SNPs.

4.2. Identification of miRNA target genes

The respective target genes of the miRNAs were identified to explicate the biological targets of these miRNAs whereby they control gene expression and ultimately regulate the cellular and molecular responses during disease development and progression. The following datasheet comprises of the potential miRNA targets genes associated with vitiligo as identified by TargetScanHuman 7.1.

miRNA	Target Gene	Target Gene Name	Representative Transcript
hsa-miR-1	EDN1	endothelin 1	ENST00000379375.5
hsa-miR-133b	GSTP1	glutathione S-transferase pi 1	ENST0000398606.3
hsa-miR-135a	SH2B3, FBXO11, TSLP, FGFR1OP, TANK, SOD2	SH2B adaptor protein 3 , -box protein 11 , thymic s	ENST00000341259.2, ENST00000402508.1, ENST00000379706.4, ENST00000366847.4, ENST0
hsa-miR-183	ICA1	islet cell autoantigen 1, 69kDa	ENST00000402384.3
hsa-miR-190	LPP, IL2	LIM domain containing preferred translocation pa	ENST00000312675.4, ENST00000226730.4
hsa-miR-214	RERE, FOXP1, RPGRIP1L, TXNDC5, CORO2A, FGFR1OP, SE	arginine-glutamic acid dipeptide (RE) repeats, for	ENST00000337907.3, ENST00000318789.4, ENST00000262135.4, ENST00000539054.1, ENST0
hsa-miR-301b	TGFBR2, ESR1	transforming growth factor, beta receptor II (70/8	ENST00000359013.4, ENST00000440973.1
hsa-miR-30a-3p	SH2B3, HLA-DQA1, VDR, PDGFRA, CCR5, STAT4, PDE10A,	SH2B adaptor protein 3, major histocompatibility c	ENST00000341259.2, ENST00000343139.5, ENST00000395324.2, ENST00000257290.5, ENST0
hsa-miR-375	IKZF4, BACH2, CORO2A, AHR	IKAROS family zinc finger 4 (Eos), BTB and CNC hor	ENST00000262032.5, ENST00000257749.4, ENST00000343933.5, ENST0000242057.4
hsa-miR-487a	BACH2, SMOC2, ZMIZ1, PTGS2, TGFBR2, ESR1, FBXO11, P	BTB and CNC homology 1, basic leucine zipper tran	ENST00000257749.4, ENST00000356284.2, ENST00000334512.5, ENST00000367468.5, ENST0
hsa-miR-517c	BACH2, VDR, HPGDS	BTB and CNC homology 1, basic leucine zipper tran	ENST00000257749.4, ENST00000395324.2, ENST00000295256.5
hsa-miR-616	FASLG, FGFR1OP, ACP1, GABPA, IL1B, HAVCR2, SGSM3, G	Fas ligand (TNF superfamily, member 6) , FGFR1 o	ENST00000340030.3, ENST00000366847.4, ENST00000272067.6, ENST00000354828.3, ENST0
hsa-miR-211	TGFBR2	transforming growth factor, beta receptor II (70/8	ENST00000359013.4
hsa-miR-136	RPGRIP1L	RPGRIP1-like	ENST00000262135.4
hsa-miR-296	APEX1	APEX nuclease (multifunctional DNA repair enzym	ENST00000557054.1
hsa-miR-328	LPP	LIM domain containing preferred translocation pa	ENST00000312675.4
hsa-let-7c	FOXP1, TOB2, SH2B3, EDN1, IL10, FAS, FASLG, TP53, GAB	forkhead box P1 , transducer of ERBB2, 2 , SH2B ad	ENST00000318789.4, ENST00000327492.3, ENST00000341259.2, ENST00000379375.5, ENST0
hsa-miR-100	CCR5, TSLP, KCNK12, ICA1, MITF, TLR4, SOD2	chemokine (C-C motif) receptor 5 (gene/pseudog	ENST00000292303.4, ENST00000379706.4, ENST00000327876.4, ENST00000402384.3, ENST0
hsa-miR-10a	LPP, SLC1A2, RPGRIP1L, TGFBR2, ESR1, FGFR1OP, TYRP1,	LIM domain containing preferred translocation pa	ENST00000312675.4, ENST00000278379.3, ENST00000262135.4, ENST00000359013.4, ENST0
hsa-miR-1180	ZMIZ1, FANCA	zinc finger, MIZ-type containing 1, Fanconi anemia	ENST00000334512.5, ENST00000568369.1
hsa-miR-135b	FANCA, FGFR1OP, SEC14L2, SOD2	Fanconi anemia, complementation group A, FGFR1	ENST00000568369.1, ENST00000366847.4, ENST00000312932.9, ENST00000538183.2
hsa-miR-145	SMOC2, CD44, COMT, PTGS2, ESR1, FGFR1OP, GLYAT, PER	SPARC related modular calcium binding 2, , CD44 n	ENST00000356284.2, ENST00000278386.6, ENST00000361682.6, ENST00000367468.5, ENST0
hsa-miR-155	FOXP1, SLA, CTLA4, TGFBR2, FBXO11, FGF2	forkhead box P1, Src-like-adaptor , cytotoxic T-lym	ENST00000318789.4, ENST00000338087.5, ENST00000427473.2, ENST00000359013.4, ENST0
hsa-miR-194	MITF	microphthalmia-associated transcription factor	ENST0000328528.6

Figure 5: Datasheet of miRNA target genes.

4.3. miRNA-target gene network

The miRNA-target gene bipartite network represented a total of 41 miRNAs and 98 unique target genes consisting of 139 nodes and 220 edges. The target genes are considered to be connected in the network if they share a common miRNA.

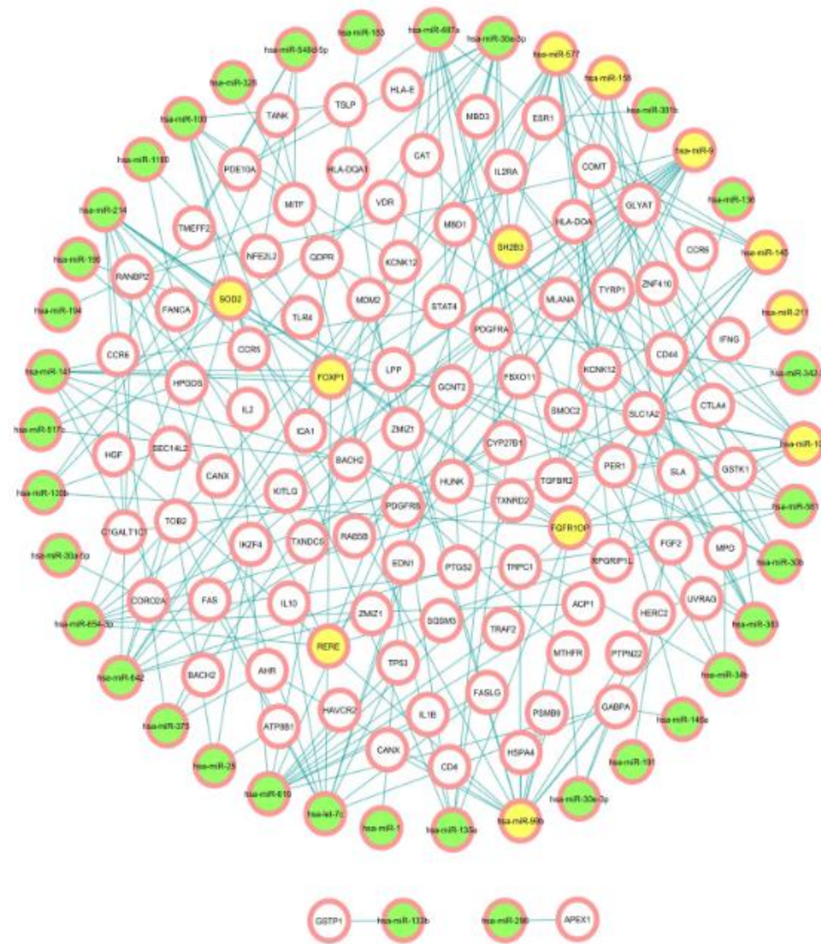

Figure 6: miRNA-target genes interaction network. The green colored nodes represent the miRNAs, the white colored nodes represents the target genes, and yellow colored nodes represent the hub miRNAs along with the target genes of the highest degree in the network.

To identify the hub miRNAs and target genes associated with vitiligo, we used cytoHubba app in Cytoscape. Among the number of methods available for hub identification, we chose Maximum Clique Centrality (MCC) for identification of hubs along with two other topological parameters, namely, betweenness centrality and bottleneck, and normalized the data to identify the top

hubs in the network. MCC is the latest and comparatively better than all other methods available in Cytoscape. In our analysis, we found 7 hub miRNAs (hsa-miR-99b, hsa-miR-577, hsa-miR-9, hsa-miR-155, hsa-miR-211, hsa-miR-10a, hsa-miR-145).

Network diameter	9
Network density	0.023
Number of nodes	139
Number of edges`	220
Clustering coefficient	0
Network heterogeneity	0.949
Average number of neighbours	3.165
Hub miRNAs	hsa-miR-99b, hsa-miR-577, hsa-miR-9, hsa-miR-155, hsa-miR-211, hsa-miR-10a, and hsa-miR-145

Table1: miRNA-target gene interaction network characteristics.

4.4. Protein-protein interaction network

Protein-protein interaction network of vitiligo associated proteins exhibited significant interconnections between the proteins. It comprised of 71 nodes and 322 edges. The proteins are considered to be connected in the network if they interact with each other. If each protein is not inclined to interact with another protein or specifically interacts with a single protein, then the bipartite network would be disconnected into many single nodes corresponding to specific or unique proteins with few or no edges between the nodes. Rather, the protein-protein interaction network generated displayed many interactions between the proteins.

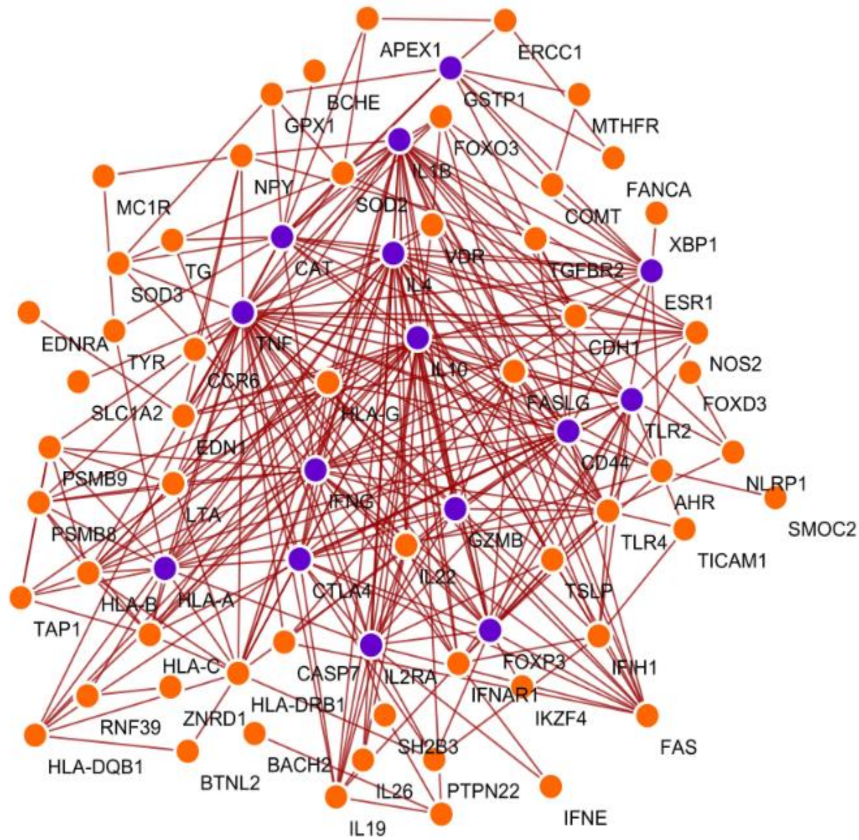

Figure 7: Protein-protein interaction network. The purple nodes represent the hub proteins in the network.

Network diameter	6
Network density	0.13
Number of nodes	71
Number of edges`	322
Clustering coefficient	0.478
Network heterogeneity	0.925
Average number of neighbours	9.07
Hub Proteins	IL10, IFNG, IL4, CD44, IL1B, CTLA4, GZMB, FOXP3, TNF, IL2RA, CAT, ESR1, TLR2, HLA-A, GSTP1

Table 2: Protein-protein interaction network characteristics.

For prioritizing proteins as hubs, we used cytoHubba app in Cytoscape to identify the hubs or essential proteins to be associated with vitiligo. We chose MCC for identification of hubs along betweenness centrality and bottleneck as topological parameters, and normalized the data to identify the top 15 hubs in the network. These 15 essential (hub) proteins are IL10, IFNG, IL4, CD44, IL1B, CTLA4, GZMB, FOXP3, TNF, IL2RA, CAT, ESR1, TLR2, HLA-A, and GSTP1.

4.5. Functional module and enrichment analysis

The functional module analysis of vitiligo associated proteins revealed 4 functional modules. The average size of the clusters was 17.75, and they were ranked by their modularity score of 1. The majority of the proteins were found to form a single large cluster. This implies that these proteins have a biological similarity in their functions.

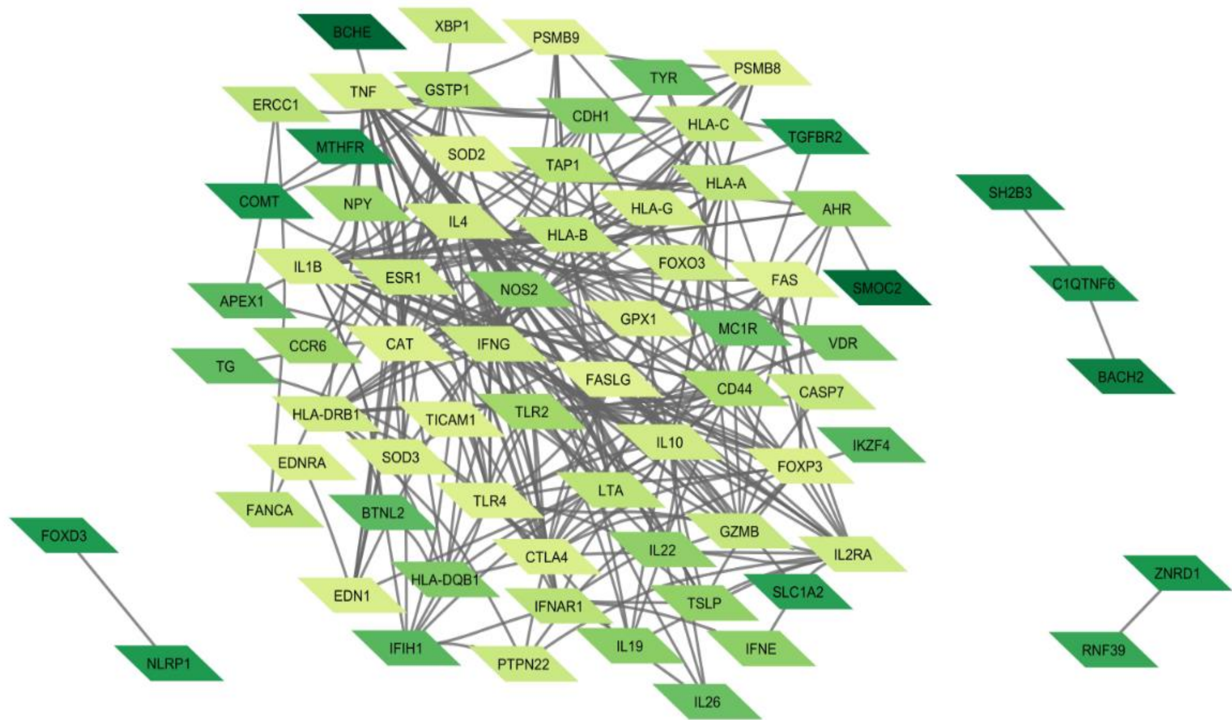

Figure 8: Functional module (clusters) network. The shades of green color, from light to dark, represent the decrease in the number of interactions. The genes with the maximum number of interactions show lighter shades in the cluster.

We performed functional enrichment analysis of the larger functional module consisting of 64 proteins using DAVID 6.8. Keeping the classification stringency at highest and considering the enrichment score value ≤1.3 to be significant, DAVID classified the given set of target genes into 9 functionally enriched clusters that involved 15 genes from the given set of miRNA target genes [Table 3].

All the 9 clusters were observed to be primarily associated with the immune system regulatory processes, such as, MHC class I/II-like antigen recognition protein, conserved site of immunoglobulin/major histocompatibility complex, conserved site of interleukin-10, positive regulation of JAK-STAT cascade, apoptotic signaling, chemokines and TNF.

Cluster 1-Enrichment Score: 6.23				
Term	Count	Genes	p-value	Benjamini
MHC class I, alpha chain, alpha1/alpha2	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	2.75E-10	5.30E-08
MHC class I-like antigen recognition	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	3.27E-08	1.58E-06
integral component of lumenal side of endoplasmic reticulum membrane	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	4.68E-08	3.23E-06
domain:Ig-like C1-type	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	4.90E-08	4.07E-06
region of interest:Connecting peptide	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	6.74E-08	4.47E-06
MHC classes I/II-like antigen recognition protein	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	3.89E-07	1.25E-05
ER to Golgi transport vesicle membrane	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	9.63E-07	3.32E-05
Immunoglobulin/major histocompatibility complex, conserved site	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	1.67E-06	3.59E-05
Viral myocarditis	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	8.85E-05	5.31E-04
Golgi membrane	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	0.053271	0.302141
Cluster 2-Enrichment Score: 5.48				
Term	Count	Genes	p-value	Benjamini
Interleukin-10, conserved site	4	IL10, IL19, IL22, IL26	7.05E-07	1.94E-05
Interleukin-10/19/20/24	4	IL10, IL19, IL22, IL26	7.05E-07	1.94E-05
positive regulation of JAK-STAT cascade	4	IL10, IL19, IL22, IL26	7.02E-05	0.002365

Cluster 3-Enrichment Score: 4.51				
Term	Count	Genes	p-value	Benjamini
MHC class II, beta chain, N-terminal	4	HLA-A, HLA-C, HLA-DQB1, HLA-DRB1	1.57E-05	3.03E-04
MHC class II, alpha/beta chain, N-terminal	4	HLA-A, HLA-C, HLA-DQB1, HLA-DRB1	4.52E-05	7.93E-04
MHC class II protein complex	4	HLA-A, HLA-C, HLA-DQB1, HLA-DRB1	5.79E-05	8.87E-04

Table 3: Functional enrichment analysis of the large cluster of 64 proteins (data shown for 3 clusters).

4.6. Ontology analysis

The gene ontology analysis results showed the distribution of 84 genes classified in the three aspects of ontology analysis which were considered, that is, biological process, molecular function, and cellular component.

4.6.1. Biological process analysis

Out of the total 84 genes, 36 genes (42.9%) were shown to take part in cellular processes (cell communication, cell cycle, cell proliferation and cellular component movement), and 26 genes (31.0%) were shown to be involved in metabolic processes (primary metabolic process, biosynthetic process, catabolic process, nitrogen compound metabolic process, and phosphate-containing compound metabolic process).

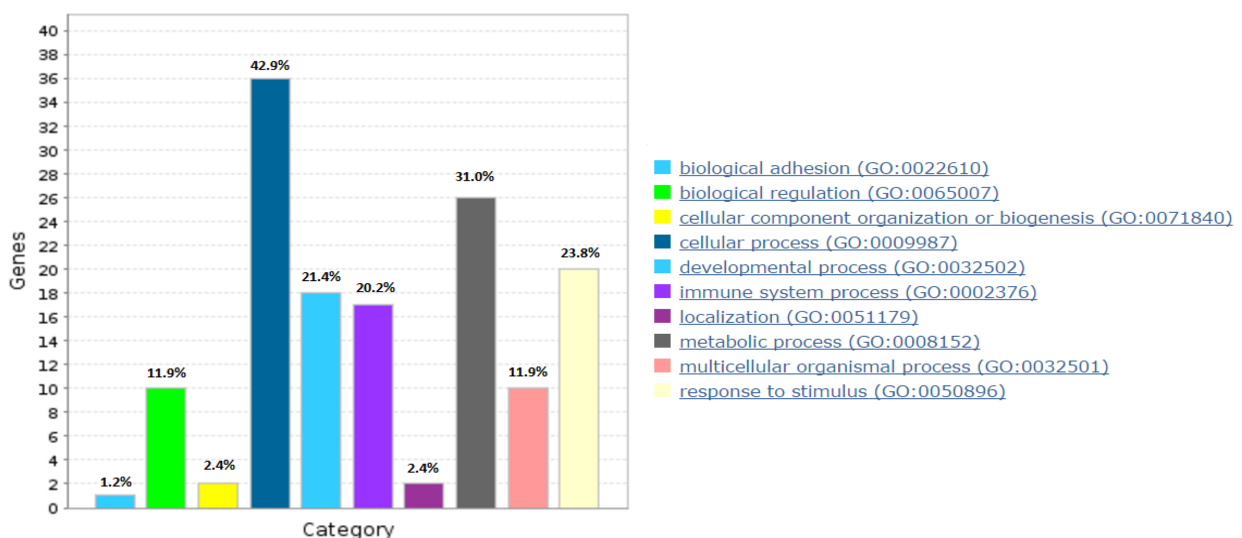

Figure 9: Bar graph of biological process analysis.

Table 4 presents the biological processes in which the genes were shown to be involved.

Category Name	Number of Genes	Genes	Percentage of Genes
cellular process (GO:0009987)	36	VDR, CAT, TNF, FASLG, ESR1, NOS2, LPP, CLNK, TLR4, SLC1A2, MTHFR, IFNE, NPY, BACH2, FOXD3, KZF4, IL1B, FOXP3, FOXO3, ATX2, CDH1, ZNRD1, CASP7, SH2B3, UVRAG, FOXP1, TLR2, SOD3, RERE, EDNRA, LTA, HERC2, AHR, FAS, NOS2, APEX1	42.90%
metabolic process (GO:0008152)	26	CAT, NLRP1, GPX1, NOS2, CAT, ERCC1, SLC1A2, FAS, IFNE, BACH2, PTPN22, GZMB, FOXD3, IKZF4, FOXP3, FOXO3, ZMIZ1, ZNRD1, FOXP1, EDNRA, HERC2, AHR, CAT, NOS2, APEX1, UBASH3A	31.00%
response to stimulus (GO:0050896)	20	CAT, TNF, FASLG, NLRP1, GPX1, NPY, HLA-DQB1, IFNAR1, IL1B, BTNL2, SH2B3, SOD3, EDNRA, IL2RA, LTA, AHR, FAS, NOS2, APEX1, HLA-DRB1	23.80%
developmental process (GO:0032502)	18	VDR, TNF, FASLG, NLRP1, NOS2, LPP, TGFBR2, IFNE, FOXD3, FOXP3, FOXO3, CDH1, BTNL2, CASP7, FOXP1, EDNRA, LTA, FAS	21.40%
immune system process (GO:0002376)	17	TNF, FASLG, NLRP1, GPX1, CLNK, IFNE, IFNG, HLA-DQB1, HLA-G, GZMB, HLA-C, SH2B3, HLA-B, IL2RA, LTA, FAS, HLA-DRB1	20.20%
biological regulation (GO:0065007)	10	CLNK, NPY, IFNAR1, IL1B, ATX2, UVRAG, EDNRA, IL2RA, FAS, NOS2	11.90%
multicellular organismal process (GO:0032501)	10	C1QTNF6, TLR4, SLC1A2, IFNE, IL1B, CDH1, TLR2, EDNRA, FAS, NOS2	11.90%

cellular component organization or biogenesis (GO:0071840)	2	ATX2, UVRAG	2.40%
localization (GO:0051179)	2	SLC1A2, UVRAG	2.40%
biological adhesion (GO:0022610)	1	TLR2	1.20%

Table 4: Biological process analysis.

4.6.2. Molecular function analysis

Out of the 84 genes, 30 genes (35.7%) were shown to participate in binding activity (protein binding, nucleic acid binding, antigen binding and calcium ion binding), and 21 genes (25.0%) were shown to be involved in catalytic activity (hydrolase activity, transferase, activity, oxidoreductase activity, ligase activity and lyase activity).

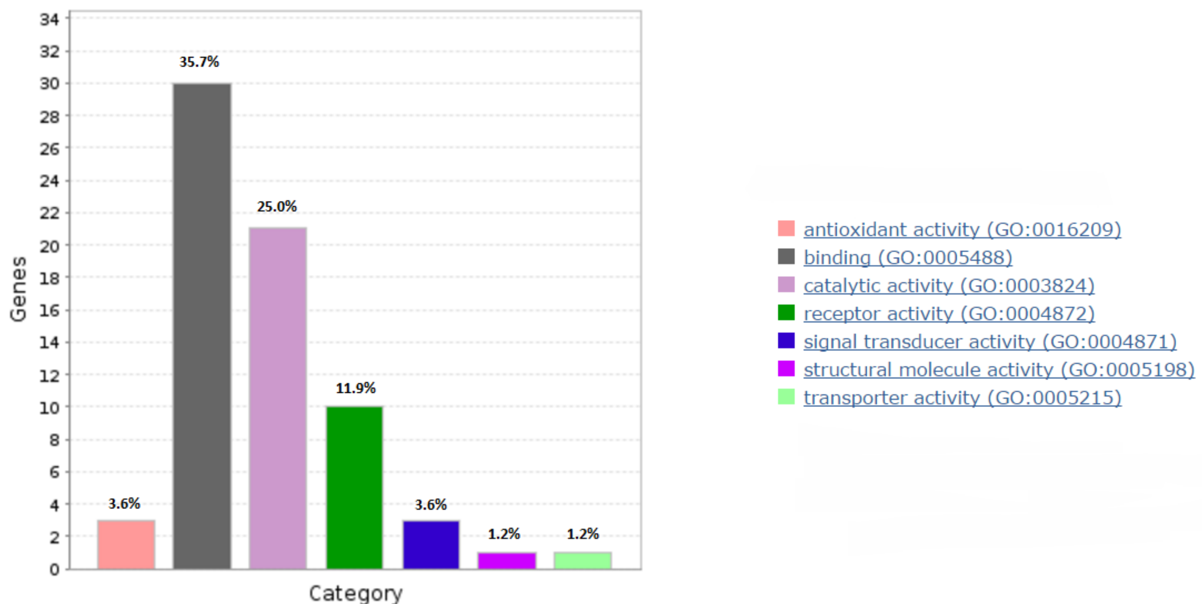

Figure 10: Bar graph of molecular function analysis.

Table 5 presents the molecular functions of the genes along with their percentage.

Category Name	Number of Genes	Genes	Percentage of Genes
binding (GO:0005488)	30	VDR, CAT, TNF, FASLG, NLRP1, ESR1, NOS2, LPP, SMOC2, ERCC1, IFNE, IFNG, NPY, HLA-G, BACH2, IFNAR1, FOXD3, HLA-C, IKZF4, IL1B, FOXP3, FOXO3, CDH1, UVRAG, HLA-B, FOXP1, SOD3, IL2RA, LTA, FAS	35.70%
catalytic activity (GO:0003824)	21	CAT, BCHE, GPX1, NOS2, LPP, CAT, ERCC1, FAS, MTHFR, COMT, BACH2, PTPN22, GZMB, ZMIZ1, ZNRD1, CASP7, SOD3, SOD2, CAT, NOS2, APEX1	25.00%
receptor activity (GO:0004872)	10	TNF, FASLG, TLR4, TGFBR2, IFNAR1, TLR2, EDNRA, IL2RA, LTA, FAS	11.90%
signal transducer activity (GO:0004871)	3	IFNAR1, IL2RA, FAS	3.60%
antioxidant activity (GO:0016209)	3	CAT, GPX1, SOD3	3.60%
structural molecule activity (GO:0005198)	1	LPP	1.20%
transporter activity (GO:0005215)	1	SLC1A2	1.20%

Table 5: Molecular function analysis.

4.6.3. Cellular component analysis

Out of the 84 genes, 19 genes (22.6%) were shown to be distributed in the cell part (intracellular part and plasma membrane), and 11 genes (13.1%) were shown to be distributed in the cell organelle (nucleus, mitochondrion, endosome, and vacuole).

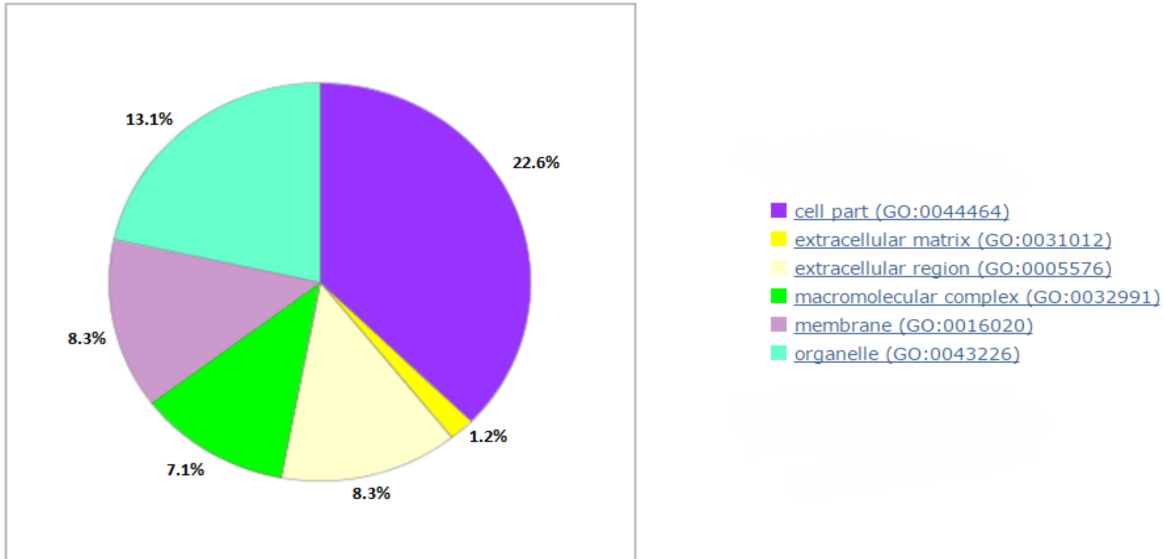

Figure 11: Pie chart of cellular component analysis.

Table 6 presents the cellular component distribution of the gene products.

Category Name	Number of Genes	Genes	Percentage of Genes
cell part (GO:0044464)	19	CAT, LPP, MTHFR, BACH2, PTPN22, GZMB, FOXD3, IKZF4, FOXP3, FOXO3, ATX2, ZNRD1, CASP7, UVRAG, FOXP1, SOD3, HERC2, AHR, FAS	22.60%
organelle (GO:0043226)	11	BACH2, GZMB, FOXD3, IKZF4, FOXP3, FOXO3, ATX2, UVRAG, FOXP1, AHR, FAS	13.10%
extracellular region (GO:0005576)	7	TLR4, IFNE, IFNG, NPY, IL1B, TLR2, SOD3	8.30%
membrane (GO:0016020)	7	LPP, HLA-G, IFNAR1, HLA-C, HLA-B, FAS, HLA-A	8.30%
macromolecular complex (GO:0032991)	6	HLA-DQB1, BACH2, ATX2, ZNRD1, AHR, HLA-DRB1	7.10%
extracellular matrix (GO:0031012)	1	TLR2	1.20%

Table 6: Cellular component analysis.

4.7. Pathway analysis

According to the threshold of hypergeometric test p≤0.001 and Benjamini-Hochberg FDR ≤0.01, the mapped genes were found to be enriched in a total of 30 pathways as listed in Table 7, which involved a total of 40 genes from the given genes set. The most significant pathway was found to be the allograft rejection pathway with a p-value of 5.70E-17 and FDR value of 1.51E-14 involving 13 genes.

Among these 30 pathways, almost half of the pathways (15 pathways) were associated with immune system responses and related disorders, and autoimmunity. While, 14 pathways were associated with infectious (viral, bacterial and parasitic) disease pathways that have been reported to impair the proper regulation of the immune system.

Term	Count	Genes	p-value	Benjamini
hsa05330:Allograft rejection	13	FAS, FASLG, GZMB, IFNG, IL10, IL4, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TNF	5.70E-17	1.51E-14
hsa04940:Type I diabetes mellitus	13	FAS, FASLG, GZMB, IFNG, IL1B, LTA, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TNF	3.29E-16	2.26E-14
hsa05332:Graft-versus-host disease	12	FAS, FASLG, GZMB, IFNG, IL1B, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TNF	8.89E-16	4.03E-14
hsa05320:Autoimmune thyroid disease	12	FAS, FASLG, GZMB, IL10, IL4, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TG	2.49E-13	8.48E-12
hsa05168:Herpes simplex infection	17	FAS, FASLG, IFNAR1, IFNG, IFIH1, IL1B, LTA, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TLR2, TICAM1, TAP1, TNF	9.20E-13	2.50E-11
hsa05321:Inflammatory bowel disease (IBD)	11	FOXP3, IFNG, IL1B, IL10, IL22, IL4, HLA-DQB1, HLA-DRB1, TLR2, TLR4, TNF	8.25E-11	1.87E-09
hsa04060:Cytokine-cytokine receptor interaction	16	CCR6, FAS, FASLG, IFNAR1, IFNE, IFNG, IL1B, IL10, IL2RA, IL22, IL26, IL4, LTA, TSLP, TGFBR2, TNF	3.61E-10	7.01E-09
hsa05140:Leishmaniasis	10	IFNG, IL1B, IL10, IL4, HLA-DQB1, HLA-DRB1, NOS2, TLR2, TLR4, TNF	5.41E-09	9.19E-08
hsa05142:Chagas disease (American trypanosomiasis)	11	FAS, FASLG, IFNG, IL1B, IL10, NOS2, TLR2, TLR4, TICAM1, TGFBR2, TNF	1.10E-08	1.66E-07

hsa04612:Antigen processing and presentation	9	IFNG, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TAP1, TNF	1.81E-07	2.47E-06
hsa05162:Measles	10	FAS, FASLG, IFNAR1, IFNG, IFIH1, IL1B, IL2RA, IL4, TLR2, TLR4	1.32E-06	1.64E-05
hsa05164:Influenza A	11	FAS, FASLG, IFNAR1, IFNG, IFIH1, IL1B, HLA-DQB1, HLA-DRB1, TLR4, TICAM1, TNF	1.45E-06	1.65E-05
hsa05143:African trypanosomiasis	6	FAS, FASLG, IFNG, IL1B, IL10, TNF	6.93E-06	7.25E-05
hsa05152:Tuberculosis	10	IFNG, IL1B, IL10, HLA-DQB1, HLA-DRB1, NOS2, TLR2, TLR4, TNF, VDR	1.40E-05	1.36E-04
hsa04630:Jak-STAT signaling pathway	9	IFNAR1, IFNE, IFNG, IL10, IL19, IL2RA, IL22, IL4, TSLP	2.45E-05	2.22E-04
hsa05133:Pertussis	7	CASP7, IL1B, IL10, NOS2, TLR4, TICAM1, TNF	3.46E-05	2.94E-04
hsa04145:Phagosome	9	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TLR2, TLR4, TAP1	3.61E-05	2.89E-04
hsa05166:HTLV-I infection	11	XBP1, IL2RA, LTA, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1, TGFBR2, TNF	4.43E-05	3.35E-04
hsa05144:Malaria	6	IFNG, IL1B, IL10, TLR2, TLR4, TNF	5.03E-05	3.60E-04
hsa05145:Toxoplasmosis	8	IFNG, IL10, HLA-DQB1, HLA-DRB1, NOS2, TLR2, TLR4, TNF	5.18E-05	3.52E-04
hsa05323:Rheumatoid arthritis	7	IFNG, IL1B, HLA-DQB1, HLA-DRB1, TLR2, TLR4, TNF	8.55E-05	5.54E-04
hsa05310:Asthma	5	IL10, IL4, HLA-DQB1, HLA-DRB1, TNF	1.04E-04	6.43E-04
hsa05416:Viral myocarditis	6	HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	1.05E-04	6.20E-04
hsa05161:Hepatitis B	8	FAS, FASLG, IFNAR1, IFIH1, TLR2, TLR4, TICAM1, TNF	1.90E-04	0.001078
hsa05146:Amoebiasis	7	IFNG, IL1B, IL10, NOS2, TLR2, TLR4, TNF	2.40E-04	0.001303
hsa04640:Hematopoietic cell lineage	6	CD44, IL1B, IL10, NOS2, TLR2, TLR4, TNF	6.87E-04	0.003589
hsa05134:Legionellosis	5	CASP7, IL1B, TLR2, TLR4, TNF	0.001034	0.005198

hsa04514:Cell adhesion molecules (CAMs)	7	CDH1, HLA-A, HLA-B, HLA-C, HLA-G, HLA-DQB1, HLA-DRB1	0.001139	0.005519
hsa04620:Toll-like receptor signaling pathway	6	IFNAR1, IL1B, TLR2, TLR4, TICAM1, TNF	0.001857	0.008679

Table 7: KEGG pathway analysis.

In the protein-pathway network, a protein is connected to a pathway if the protein is known to be involved in that particular pathway. The network consisted of 71 nodes and 270 interactions representing 40 proteins and 30 pathways.

Figure 12: Protein pathway network. The red colored nodes represent the proteins, the dark blue colored nodes represents the pathways, and the light blue colored nodes represents the hub proteins.

Based on degree value, TNF showed maximum interaction in the network implying its participation in most of the pathways. Among the 30 pathways, TNF was found to be associated with 24 pathways. Similarly, IL1B, IFNG, IL10, and TLR2 were found to be involved in 19, 18, 15 and 14 pathways respectively. It was also observed that TLR2 was specifically associated with the infectious disease pathways that were responsible for deregulating the immune system processes as depicted in the results of KEGG pathway analysis.

Table 8 lists the pathways in which the hub proteins are involved. Among the 15 hub proteins identified in the protein-protein interaction network, 11 were found in this network suggesting that these 11 essential proteins are involved in the filtered significant pathways.

Hub Proteins	Pathways	Number of Pathways
TNF	Allograft rejection, Type I diabetes mellitus, Graft-versus-host disease, Herpes simplex infection, Inflammatory bowel disease (IBD), Cytokine-cytokine receptor interaction, Leishmaniasis, Chagas disease (American trypanosomiasis), Antigen processing and presentation, Influenza A, African trypanosomiasis, Tuberculosis, Pertussis, HTLV-I infection, Malaria, Toxoplasmosis, Rheumatoid arthritis, Asthma, Hepatitis B, Amoebiasis, Hematopoietic cell lineage, Legionellosis, Toll-like receptor signaling pathway, TNF signaling pathway	24
IL1B	Type I diabetes mellitus, Graft-versus-host disease, Herpes simplex infection, Inflammatory bowel disease (IBD), Cytokine-cytokine receptor interaction, Leishmaniasis, Chagas disease (American trypanosomiasis), Measles, Influenza A, African trypanosomiasis, Tuberculosis, Pertussis, Malaria, Rheumatoid arthritis, Amoebiasis, Hematopoietic cell lineage, Legionellosis, Toll-like receptor signaling pathway, TNF signaling pathway	19
IFNG	Allograft rejection, Type I diabetes mellitus, Graft-versus-host disease, Herpes simplex infection, Inflammatory bowel disease (IBD), Cytokine-cytokine receptor interaction, Leishmaniasis, Chagas disease (American trypanosomiasis), Antigen processing and presentation, Measles, Influenza A, African trypanosomiasis, Tuberculosis, Jak-STAT signaling pathway, Malaria, Toxoplasmosis, Rheumatoid arthritis, Amoebiasis	18

TLR2	Herpes simplex infection, Inflammatory bowel disease (IBD), Leishmaniasis, Chagas disease (American trypanosomiasis), Measles, Tuberculosis, Phagosome, Malaria, Toxoplasmosis, Rheumatoid arthritis, Hepatitis B, Amoebiasis, Hematopoietic cell lineage, Legionellosis, Toll-like receptor signaling pathway	15
IL10	Autoimmune thyroid disease, Inflammatory bowel disease (IBD), Cytokine-cytokine receptor interaction, Leishmaniasis, Chagas disease (American trypanosomiasis), African trypanosomiasis, Tuberculosis, Jak-STAT signaling pathway, Pertussis, Malaria, Toxoplasmosis, Asthma, Amoebiasis, Hematopoietic cell lineage	14
IL4	Autoimmune thyroid disease, Inflammatory bowel disease (IBD), Cytokine-cytokine receptor interaction, Leishmaniasis, Measles, Jak-STAT signaling pathway, Asthma	7
HLA-A	Allograft rejection, Type I diabetes mellitus, Graft-versus-host disease, Autoimmune thyroid disease, Herpes simplex infection, Antigen processing and presentation, Phagosome, HTLV-I infection, Viral myocarditis, Cell adhesion molecules (CAMs)	10
IL2RA	Cytokine-cytokine receptor interaction, Measles, Jak-STAT signaling pathway, HTLV-I infection	4
GZMB	Type I diabetes mellitus, Graft-versus-host disease, Autoimmune thyroid disease	3
CD44	Hematopoietic cell lineage	1
FOXP3	Inflammatory bowel disease (IBD)	1

Table 8: The pathways of the hub proteins in the protein-pathway network.

4.8. SNP analysis

Among the 134 SNPs, 36 (26.9%) are nsSNPs and 7 (5.2%) are sSNPs, while, 68 (50.8%) SNPs were found in the non-coding region. SNPs in the non-coding region comprises of 44 (32.8%) SNPs in the intronic region, 19 (14.2%) in the near-gene region, and 5 (3.8%) in the mRNA UTR region. Rest 23 (17.1%) among 134 SNPs are intergenic. The distribution of the SNPs is shown in Figure 13.

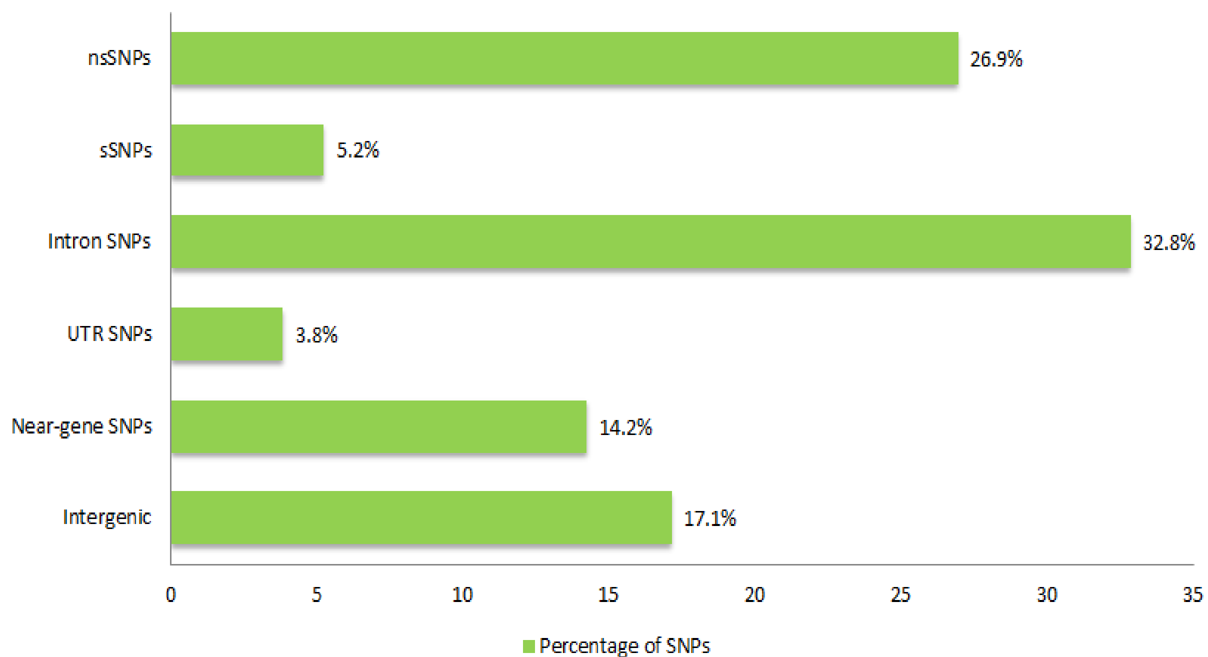

Figure 13: Graph of the distribution of SNPs.

For our analysis, we selected the nsSNPs and UTR-region SNPs since UTRs are central for the post-transcriptional regulation of gene expression and alterations in the functional UTR region can lead to serious pathology (Conne et al., 2000). The 36 nsSNPs were analyzed by using a combination of SIFT, PolyPhen, PROVEAN, SNPs&GO, I-Mutant Suite and PANTHER Evolutionary Analysis of Coding SNP tools.

Table 9 and 10 presents the deleterious SNPs obtained through the SIFT, PolyPhen, PROVEAN, SNPs&GO, I-Mutant Suite and PANTHER Evolutionary Analysis of Coding SNP analysis of the vitiligo associated nsSNPs.

SNP	Genes	SIFT Score	SIFT Prediction	PolyPhen Score	PolyPhen Prediction	PROVEAN Score	PROVEAN Prediction
rs1801133	MTHFR	0.053	DELETERIOUS	0.998	probably damaging	0.002	Damaging
rs5743708	TLR2	0.016	DELETERIOUS	1	possibly damaging	0	Damaging
rs11575993	SOD2	0.014	DELETERIOUS	1	possibly damaging	0.001	Damaging

Table 9: nsSNPs found to be deleterious by SIFT, PolyPhen, and PROVEAN.

SNP	Genes	I MUTANT Score	I MUTANT Prediction	SNPs&GO Score	SNPs&GO Prediction	PANTHER
rs1801133	MTHFR	-0.78	Disease-Related Mutation	0.88	Disease associated variation	probably damaging
rs5743708	TLR2	-2.78	Disease-Related Mutation	0.7	Disease associated variation	possibly damaging
rs11575993	SOD2	-1.4	Disease-Related Mutation	0.66	Disease associated variation	probably damaging

Table 10: nsSNPs found to be deleterious by SNPs&GO, I-Mutant Suite and PANTHER.

SIFT predicted 6 nsSNPs (16.7%) to be deleterious with a tolerance score cut off which is ≤0.05. Further analysis of the nsSNPs using PolyPhen predicted 2 nsSNPs to be “probably damaging”, and 5 nsSNPs to be “possibly damaging” with a tolerance cut off score ≥0.5. Consequently, 7 nsSNPs (19.4%) were characterized as damaging.

Analysis using PROVEAN revealed a similar result as that of Polyphen tool. Based on a tolerance cut off score value ≤0.05, it predicted 7 nsSNPs (19.4%) to be damaging. Of these 7 nsSNPs, one (rs5743708) was reported to be highly damaging with a tolerance score of 0.

To improve the prediction accuracy, we used I-Mutant Suite and SNPs&GO tool for further analysis. In the I-Mutant Suite results, we found that 11 nsSNPs (30.6%) exhibited a DDG value of less than -0.5, which indicates that these are largely unstable resulting in disease-associated

mutations. Whereas, SNPs&GO predicted 10 nsSNPs (27.8%) to be related to disease-associated mutations by using a tolerance cut off score ≥0.5.

A disease causing mutation in the highly conserved regions of the genome may pose a high risk of that particular mutation to be damaging which is why we decided to carry out SNP conservation analysis. The probability of a mutation to cause a disease increases monotonically with the increase in the degree of site conservation (Vitkup et al., 2003). Conservation analysis by PANTHER Evolutionary Analysis of Coding SNP predicted 14 nsSNPs (38.9%) to be deleterious based on their preservation time.

Since different in silico tools have a diverse set of alignments and molecular characteristics, the results of the six tools were slightly different. Accordingly, we combined the results of SIFT, PolyPhen, PROVEAN, SNPs&GO, I-Mutant Suite and PANTHER Evolutionary Analysis of Coding SNP to predict the deleterious nsSNPs common in all the analysis.

Figure 14 shows the distribution of deleterious and benign nsSNPs obtained using SIFT, PolyPhen, and I-Mutant Suite.

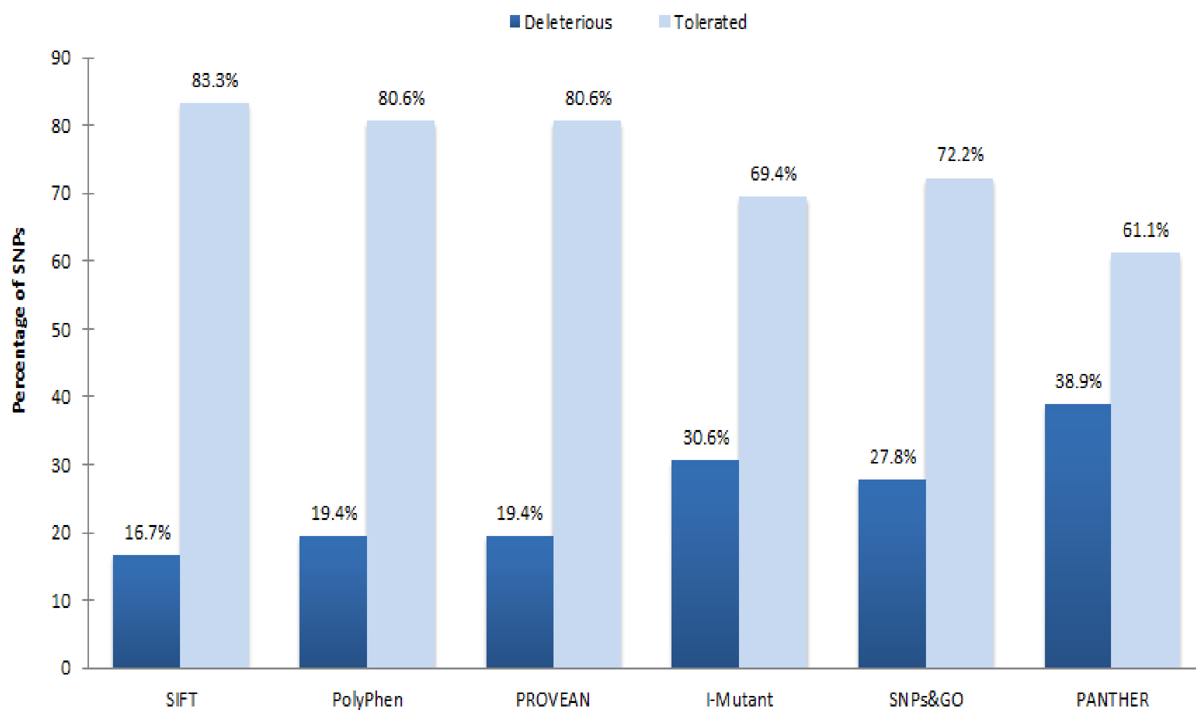

Figure 14: Graph of the distribution of the deleterious and benign nsSNPs as predicted by SIFT, PolyPhen, PROVEAN, I-Mutant Suite, SNPs&GO, and PANTHER.

Of all of the predictions, 16.7%, 19.4%, 19.4%, 30.6%, 27.8% and 38.9% deleterious nsSNPs were specifically found by SIFT, PolyPhen, PROVEAN, SNPs&GO, I-Mutant Suite and PANTHER

Evolutionary Analysis of Coding SNP respectively. Combining the results of all the six tools, three nsSNPs, namely, rs1801133 (MTHFR), rs5743708 (TLR2) and rs11575993 (SOD2) were predicted to be functionally significant.

MutPred predicted the molecular cause of the nsSNPs to become deleterious in MTHFR (rs1801133), TLR2 (rs5743708) and SOD2 (rs11575993) [Table 12]. Analysis of the results showed an interrelation of the SNPs to be damaging with the solvent accessibility of the protein. The type of mutated residue and its position in the sequence affect the stability of the protein and the stability of the protein due to mutation decreases with the decrease in solvent accessibility of a residue (Vitkup et al., 2003).

Genes	Amino Acid Substitution	MutPred Score	Molecular Mechanisms with p-values ≤ 0.05
MTHFR	A222V	0.831	Loss of relative solvent accessibility (P = 8.3e-03) Altered ordered interface (P = 0.03) Loss of helix (P = 0.03) Altered metal binding (P = 0.01) Gain of strand (P = 0.04) Loss of allosteric site at F224 (P = 0.02) Altered transmembrane protein (P = 0.01)
TLR2	R753Q	0.518	Gain of relative solvent accessibility (P = 0.03) Altered transmembrane protein (P = 5.8e-04) Altered ligand binding (P = 0.01)
SOD2	L84F	0.712	Altered ordered interface (P = 0.01) Altered transmembrane protein (P = 9.6e-04) Loss of relative solvent accessibility (P = 0.03) Altered DNA binding (P = 0.03)

Table 11: Mutational analysis by MutPred.

NetSurfP predicted the surface solvent accessibility of amino acids by using the protein FASTA sequence as a query. The solvent accessibility is predicted to be buried or exposed, based on the accessibility of the amino acid residues to the solvent. The reliability of relative surface accessibility is verified in the form of Z-score which highlights the surface prediction reliability.

As given in Table 13, the class assignment does not change for the 3 nsSNPs. Although there were very minimal changes in the Relative Surface Accessibility (RSA) values for the 3 nsSNPs, a

considerable drift in the Z-score was not observed between the wild type and mutant type proteins.

Genes	Type	Class assignment	Relative Surface Accessibility (RSA)	Z-fit score for RSA prediction
MTHFR	Wild	B	0.027	0.617
	Mutant	B	0.026	0.781
TLR2	Wild	E	0.244	1.46
	Mutant	E	0.243	1.527
SOD2	Wild	B	0.2	-0.736
	Mutant	B	0.166	-0.705

Table 12: Solvent accessibility analysis of the mutated proteins by NetSurfP.

UTRScan results predicted 2 UTR SNPs to cause functional pattern change after comparing the functional patterns of each UTR SNP. The 2 SNPs, namely, rs1129038 (HERC2) and rs10768122 (SLC1A2) exhibited a functional pattern change of the upstream open reading frame (uORF). Alterations in the uORF region within the disease-associated genes have been reported to silence the expression of the downstream ORF influencing the protein expression which in turn influences human phenotype and disease (Calvo et al., 2009).

Further analysis of the 3'UTR SNPs using PolymiRTS predicted rs1129038, rs10768122, and rs4946936 to profoundly affect the microRNA binding target sites in HERC2, SLC1A2, and FOXO3 mRNA transcripts respectively.

Genes	rs ID	PolymiRTS miR ID	Function Class
HERC2	rs1129038	hsa-miR-3194-3p, hsa-miR-5691 , hsa-miR-6805-3p	C
SLC1A2	rs10768122	hsa-miR-369-3p, hsa-miR-374a, hsa-miR-374b	D
FOXO3	rs4946936	hsa-miR-548av-3p	D

Table 13: SNP analysis of 3'UTRs by polymiRTS.

4.9. Drug-target network

The bipartite network of drug–protein target interaction consisted of 109 nodes and 84 interactions. A drug and protein are considered to be connected to each other if the protein is a known target of the drug, giving rise to a drug–target network.

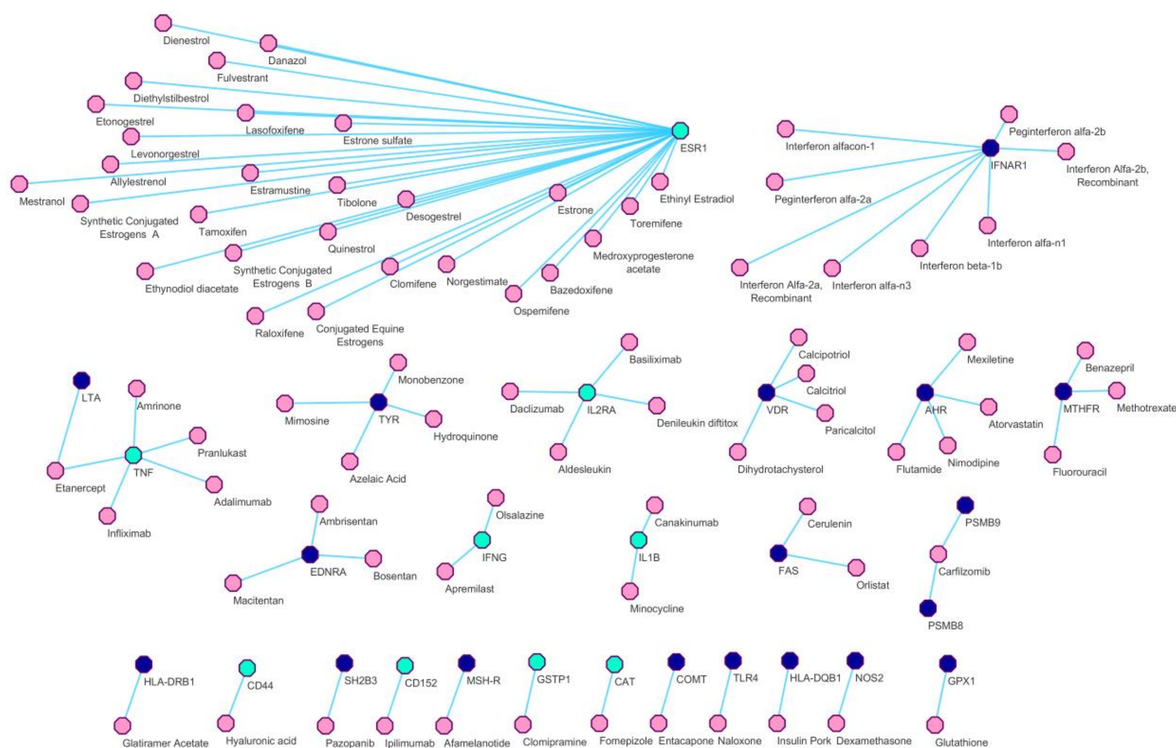

Figure 15: Drug–target network. The cyan colored nodes represents the hub protein targets, the dark blue colored nodes represents the protein targets, and the pink colored nodes represents the drugs.

Network diameter	3
Network density	0.014
Number of nodes	109
Number of edges`	84
Clustering coefficient	0
Network heterogeneity	1.77
Average number of neighbours	1.541

Table 14: Drug-target network characteristics.

In our analysis, we found that the most of the drugs targeting a particular protein did not show any interaction with other protein targets in the network except for the two drugs, Etanercept and Carfilzomib. Etanercept targets both TNF and LTA while Carfilzomib targets PSMB8 as well as PSMB9. Also, we found that out of the 15 hub proteins, only 9 protein targets were found to be present in this network. This illustrates the other 6 hub proteins (IL10, IL4, GZMB, FOXP3, TLR2, AND HLA-A) as potential drug candidates for which drug information is currently not available. Also, we found that 4 hub proteins, namely, CD44, CD152, CAT, and GSTP1 were targeted by a single drug. This highlights the imperative need to discover more effective drugs that target these proteins which may play a major role in therapeutics to alleviate disease conditions in patients.

Another notable finding in our analysis was that the drugs which showed a high degree in the network were mostly indicated for the treatment of autoimmune diseases and deregulated immune responses. One of them is Etanercept that targets TNF, a major proinflammatory cytokine that affects various aspects of the immune response. Etanercept is a genetically engineered decoy receptor that consists of the ligand-binding domain of TNFR2 and the Fc component of human IgG1. It competitively binds with high affinity to TNFR2 inhibiting the binding of both TNF-α and TNF-β to the cell surface receptors, consequently, inhibiting inflammation induced melanocyte death. It has been indicated to be clinically used for rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and Crohn's disease (Nanda and Bathon, 2004). However, Etanercept has been reported to be less efficient as a monotherapy in vitiligo patients requiring the need of a combinative therapy (Rigopoulos et al., 2007).

LTA is involved in the follicular dendritic cells development and has been observed to induce signals leading to lymphoid neo-organogenesis driving the inflammatory responses in autoimmune diseases like rheumatoid arthritis (Takemura et al., 2001). This suggests that etanercept may suppress lymphoid neo-organogenesis and reduce the proliferation of mature dendritic cells in vitiligo lesions (Wang et al., 2011).

The other drug, Carfilzomib, is a tetrapeptide epoxy ketone based proteasome inhibitor that targets PSMB9 and PSMB8. Peptides generated from ubiquitin-tagged cytosolic proteins are presented to CTLs by MHC class-I molecules which are degraded by multi-catalytic, cytosolic immune-proteasome complex called LMP2 and LMP7 encoded by PSMB9 and PSMB8 genes respectively (Cresswell et al., 2005). This intrinsic enzymatic activity of immune-proteasomes may be altered by genetic variations which reduce the expression of PSMB8 and PSMB9 in vitiligo PBMCs after IFNG stimulation. This leads to defective proteolytic degradation and accumulation of ubiquitinylated proteins in the epidermis of vitiligo patients leading to ROS production and auto-inflammatory immune responses which may be detrimental for the manifestation of vitiligo (Dani et al., 2017). Carfilzomib targets the catalytic activity of immune-

proteasomes and irreversibly inactivates the proteasome thereby, inhibiting aberrant immune function (Miller et al., 2013).

4.10. Drug-similarity network

The drug similarity tripartite network of protein targets, drugs and interacting drugs comprised of 178 nodes and 1331 interactions. A drug and its interacting drug are considered to be connected if they share a common protein target. Interacting drug partners of a particular drug may enhance the efficacy of the drug or may even target the same protein. Such interacting drugs may represent themselves as potential drug repositioning candidates. With this concept, we constructed the drug-similarity network that displayed interconnections between drugs and their interacting drug partners.

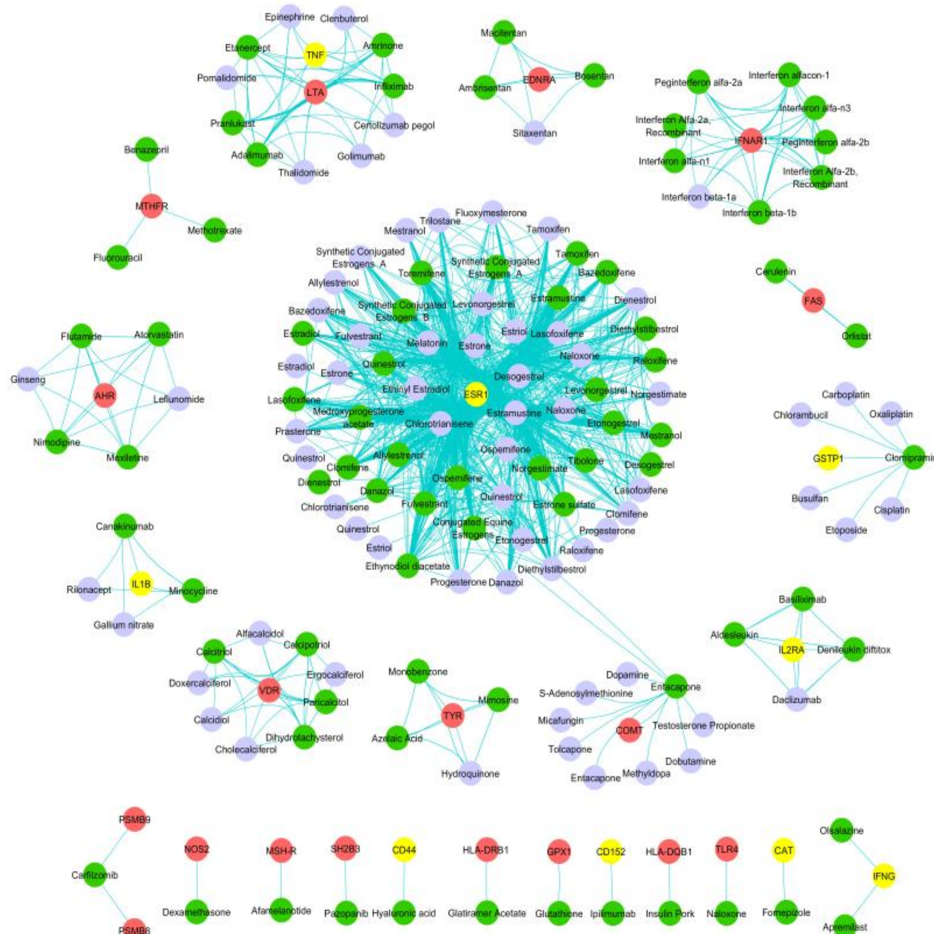

Figure 16: Drug-similarity network. The pink colored nodes represents the hub protein targets, the yellow colored nodes represents the protein targets, the green colored nodes represents the drugs, and the blue colored nodes represent the interacting drugs.

Network diameter	4
Network density	0.076
Number of nodes	178
Number of edges`	1331
Clustering coefficient	0.534
Network heterogeneity	1.213
Average number of neighbours	13.517

Table 15: Drug similarity network characteristics.

Apart from the drugs Etanercept and Carfilzomib targeting more than one protein as shown in the drug-target network, we found two other drugs showing interaction with another protein target in the network. Both Diethylstilbestrol and Conjugated equine estrogens targeting ESR1, which is one of the hub protein identified in the protein-protein interaction network analysis, were found to be interacting with the drug Entacapone targeting COMT. This signifies that the two drugs interacting with Entacapone might target COMT which is targeted by Entacapone alone with no interacting drugs reported yet. Also, we found that there are no interacting drugs reported for Etanercept targeting LTA and our drug similarity network analysis suggests that the interacting drugs for Etanercept targeting TNF might as well target LTA.

Etanercept has been less efficient as a monotherapy as mentioned earlier; therefore the interacting drugs for Etanercept as shown in the network might catalyze its efficacy when used in combination. Thus, further comprehensive study is required to validate the effectiveness of these drugs in combinative therapy.

In addition, GSTP1, also a hub protein, is targeted by a single drug (Clomipramine), but 7 interacting drugs were shown to be connected to this drug in the network. These interacting drugs can be further studied to investigate their potential as drug repositioning candidates for vitiligo treatment.

4.11. Protein modeling of TLR2

The G23D tool generated a full-length protein model for the TLR2 protein based on E-value which was 0.001 by default as generated from the PDB library and ModBase hits. Since we did not perform any molecular dynamics simulation studies for protein structure optimization, therefore, the 3D homology model generated by G23D is a preliminary model implicating the disruptive role of the SNP (rs5743708) on TLR2 protein.

Figure 17 shows the cartoon representation of the modeled protein structure including both the wild-type (green) and the mutant (light blue) amino acid. The backbone, which is the same for each amino acid, is colored gray. It also displayed the other variants as reported in ClinVar, COSMIC, and dbSNP for TLR2 which is colored as red, yellow and dark green respectively in stick representation in the 3D structure.

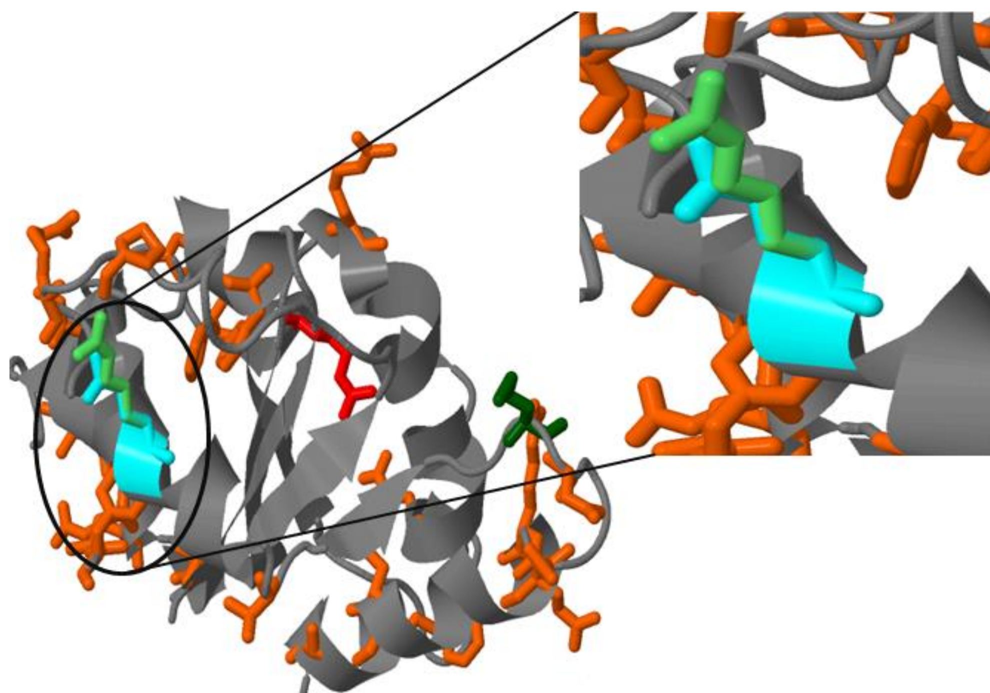

Figure 17: 3D structure of modeled mutant (R753Q) TLR2 protein. The wild type residue is represented in green color and the mutant residue is colored cyan. Other variants are also displayed in this structure with the dbSNP variant colored dark green, COSMIC variants colored orange, and ClinVar variant is represented in red color. The backbone of the protein is shown in gray color.

An A/G mutation (rs5743708) caused the substitution of the amino acid from Arginine into Glutamine at position 753 (R753Q) of the protein. The mutation was identified to be located within the TIR domain as annotated in UniProt. Since it is a membrane protein, it is exposed on the surface which is in agreement with the previous NetSurfP results.

Analysis of the 3D structure suggests that the residue glutamine in position 753 is located in the middle of a helix. There was a difference in the size observed in the wild-type and mutant amino acids with the mutant residue being smaller than the wild-type residue causing conformational changes on the DD loop which might lead to loss of interactions. Also, the wild-type residue is positively charged, but the amino acid substitution leads to a decrease in the positive charge that changes the interaction surface within the TIR domain via altered electrostatic potential. These may affect TLR2 dimerization causing loss of interactions with other molecules or residues affecting the functional activity of the protein (Xiong et al., 2012).

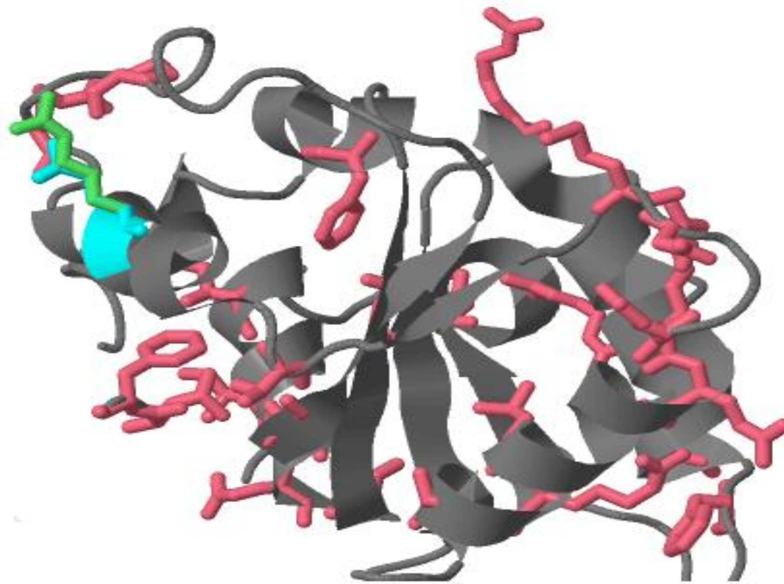

Figure 18: TIR domain in TLR2 protein. The TIR domain is represented in pink color, while the wild type and mutant residue in the domain is represented in green and cyan colors respectively.

The R753Q polymorphism compromises the TLR2/1 or TLR2/6 assembly resulting in deficient tyrosine phosphorylation and impaired recruitment of Myd88. This reduces the phosphorylation of IRAK1 and diminishes the activation of MAPKs and NF-κB resulting in the deficient production of cytokines thus altering TLR2 signaling competence. The reduced activation of NF-κB signaling pathway results in melanocyte apoptosis suggesting their decisive role in the increased risk for the development of vitiligo (Karaca et al., 2013; Traks et al., 2015).

The protein-protein interaction analysis of TLR2 showed its interaction with TLR1, TLR6, LY96, MyD88, IRAK4, TRAF6, NFKB1, TIRAP, CD14, and HMGB1. All these proteins were found to have a fundamental role in regulating the innate immune responses.

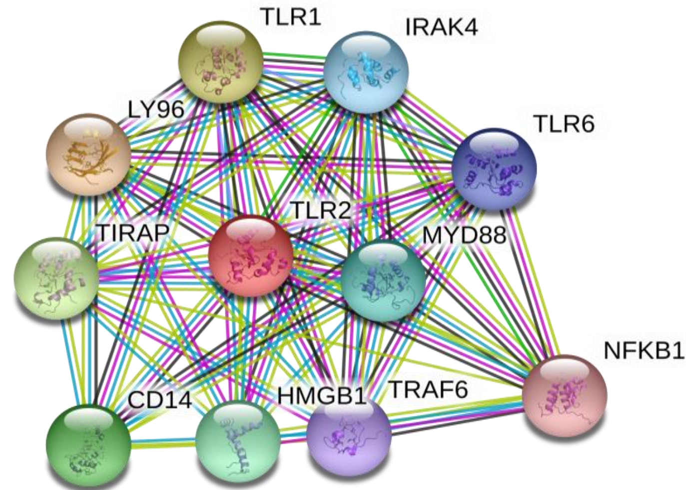

Figure 19: PPI network of TLR2.

The amino acid substitution of arginine to glutamine at position 753 has been reported to be located in the TIR domain. The TIR domain of TIRAP binds the TIR domain of TLR2 which then recruits MyD88. MyD88 facilitates the recruitment of IRAK4 which activates TRAF6 leading to the translocation of activated NF-κB to the nucleus where it induces target gene expression generating inflammatory responses (Oliveira-Nascimento et al., 2012). Mutations in the TIR domain tend to have more severe impact on signaling than those affecting the extracellular domain (Karaca et al., 2013). This implicates the possible detrimental effect of this mutation on the interaction of TLR2 with TIRAP, MyD88, IRAK4, TRAF6 and the consequent signal transduction.

TLR2 activates NF-κB in combination with either TLR1 or TLR6 by bringing together the TIR domains and triggering tyrosine phosphorylation whereby it extends a docking platform for MyD88 recruitment. Genetic alterations in TLR2 might affect its interaction with both TLR1 and TLR6 consequently affecting NF-κB activation (Brown et al., 2006). It was also found that this mutation significantly reduced NF-κB activation by about 50 and 75% (Merx et al., 2007; Ben-Ali et al., 2011). In the case of vitiligo, altered NF-kB signaling results in impaired melanogenesis inciting human melanocytes susceptible to TNF-α-induced apoptosis (Shang et al., 2002).

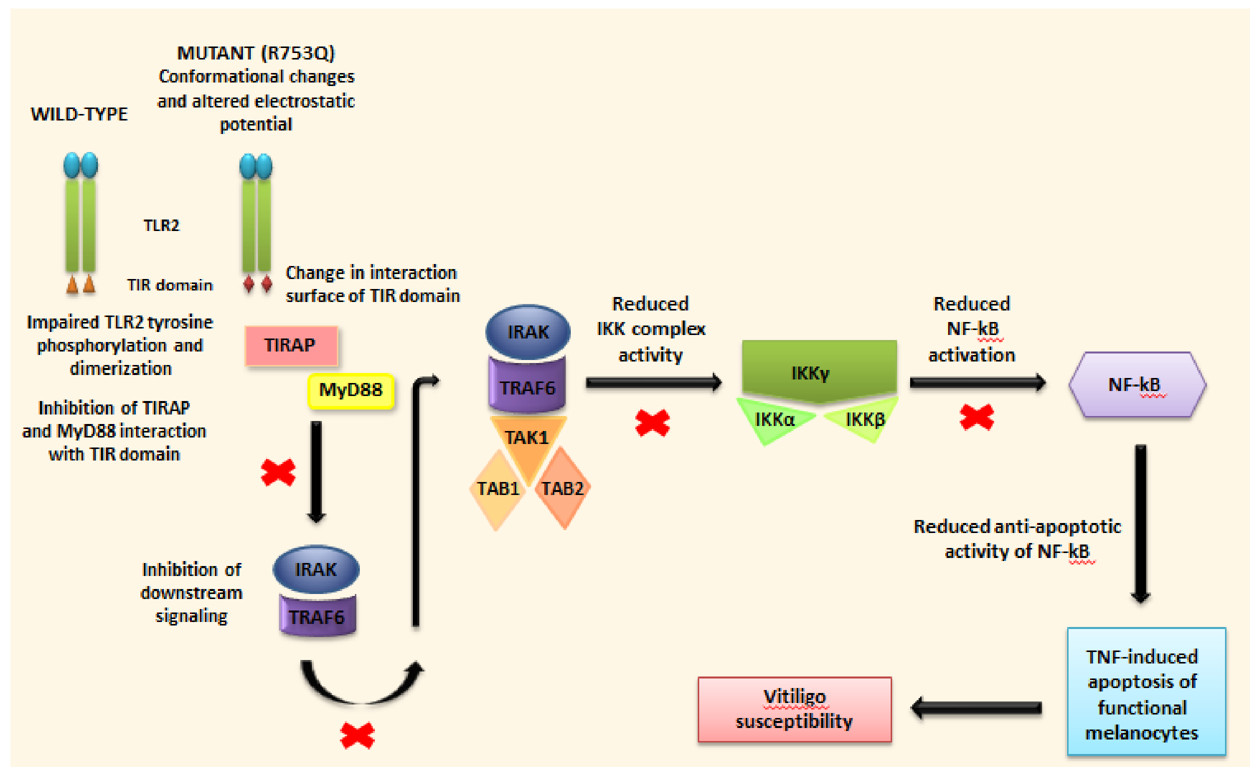

Figure 20: Disease mechanism influenced by R753Q TLR2 polymorphism.

HMGB1 has been found to upregulate the expression of TLR2 of the NK cells and promote NK cell activation mediating inflammatory responses (Qiu et al., 2014), while, CD14 (a co-receptor of TLR2) accelerates the microbial ligand transfer from CD14 to TLR2, resulting in an increased TLR2 signaling (Raby et al., 2013).

LY96 have been found to enhance the expression of both TLR2 and TLR4 and vice-versa enabling them to generate highly sensitive responses to a broad range of microbial lipopolysaccharide (LPS) structures (Dziarski et al., 2001). Altered expression of TLR2 will invariably affect the interaction of TLR2 with these proteins thereby influencing TLR2 signaling and inflammatory responses resulting in defective immune response to some antigens such as viruses in the case of vitiligo (Karaca et al., 2013).

CHAPTER 5

This chapter discusses the main results of the present work.

DISCUSSION

Vitiligo is a result of convoluted interactions of biological, environmental and immunological events; hence, a single concept cannot be attributable to all the conditions of functional melanocyte loss. Intense studies and characterization of miRNAs have elucidated their interpretative functions in growth and development, in the transformation of cellular responses to extracellular signals facilitating signal transduction. Having a cardinal role in maintaining physiological homeostasis and disease development and progression, miRNAs are significant for melanocyte development and survival (Mansuri et al., 2016). Most of the vitiligo associated genes are plausible biological candidate genes that are responsible for stimulating melanocyte-specific immune response. These candidate genes encode immunoregulatory and melanocyte proteins constituting a dense immunoregulatory network that highlights the systems and pathways mediating vitiligo susceptibility (Spritz, 2013). Network-based studies of these interacting proteins may impart an insight into disease pathogenesis initiating better diagnosis and the feasibility of personalized treatment for vitiligo patients in the future.

Our miRNA-target genes network analysis revealed 7 hub miRNAs, namely, hsa-miR-99b, hsa-miR-577, hsa-miR-9, hsa-miR-155, hsa-miR-211, hsa-miR-10a and hsa-miR-145 implicating their role in vitiligo pathogenesis. The upregulation of hsa-miR-99b reduces the cytotoxic activity (cytokine effector functions) of NK cells which are crucial for the normal BCR signaling and proliferation of B-cells. This causes deregulation of genes involved in B-cell maturation and development resulting in the dysfunctioning of the immune system indicating them to be important players in vitiligo immunopathogenesis (Nandgopal et al., 2014; Šahmatova et al., 2016).

TYRP1 is targeted by hsa-miR-577, and its reduced expression as induced by miR-577 leads to increased sensitivity of melanocytes to oxidative stress causing early cell death of vitiligo melanocytes (Manga et al., 2006; Sturm and Duffy, 2012). Also, the down-regulation of PTPN22 was observed to be influenced by miR-577 which triggers the overexpression of T-cells and suppresses anti-apoptotic AKT kinase inducing melanocyte destruction, thereby, rendering susceptibility to autoimmunity in vitiligo patients (Mansuri et al., 2016). Elevated levels of SIRT1 have been reported to protect cells from oxidative stress and inflammatory microenvironment (Han et al., 2008). Increased expression of miR-9 downregulates SIRT1 resulting in melanocytes apoptosis in vitiligo (Saunders et al., 2010). SIRT1 has been shown to regulate stress-activated MAPK pathway via Akt and ASK1 in vitiligo keratinocytes (Becatti et al.,

2014). Previous studies have demonstrated the influence of miR-145 on the genes involved in the pigmentation process (Dynoodt et al., 2013). The genes targeted by miR-145 also regulates MAPK pathway along with JNK and TGFB signaling pathway and are related to the functional groups that might indirectly influence cellular processes in vitiligo wherefore they interfere with melanocytes function and viability (Šahmatova et al., 2016) suggesting the role of both miR-9 and miR-145 in the destruction of melanocytes in vitiligo.

TGFBR2 is targeted by miR-211 and downregulated miR-211 increases the expression of TGFBR2 which in turn downregulates MITF. MITF is a known primary regulator of melanocyte development and its survival (Levy et al., 2010), thus, deregulated MITF will considerably affect melanocyte development implying the role of miR-211 in vitiligo pathogenesis. Overexpression of miR-155 was found to modulate the levels of several interferon-regulated genes, such as SOCS1, IFITM1 and IRF1 that inhibits the expression of melanogenesis associated genes, such as, TYRP1, YWHAE, SDCBP and SOX10 in melanocytes and particularly YWHAE in keratinocytes. This suggests that upregulated miR-155 is associated with vitiligo pathogenesis which alters interferon signaling as well as targets melanogenesis associated genes (Šahmatova et al., 2016). Both miR-155 and miR-10a are on their own largely dispensable for regulatory T cell (Treg) function and stability which is responsible for suppressing autoimmune pathology. Inhibition of miR-10a expression leads to reduced FOXP3 expression levels which subsequently decrease the stability of Treg cells (Jeker et al., 2012) resulting in the insufficient suppression of inflammation in autoimmune diseases which could likely happen in vitiligo patients.

Genetic variation alters or damages protein structure disrupting protein-protein interactions which are otherwise essential for regulatory processes constituting the pretext of disease development. Our PPI network analysis results identified 15 hub proteins, namely, IL10, IFNG, IL4, CD44, IL1B, CTLA4, GZMB, FOXP3, TNF, IL2RA, CAT, ESR1, TLR2, HLA-A, and GSTP1 to be associated with vitiligo. The balance between pro and anti-inflammatory cytokines plays a significant role in the pathogenesis of vitiligo. Higher concentrations of IFNG, a pro-inflammatory cytokine enhances T cell-melanocyte attachment in the skin initiating T cell mediated apoptosis of melanocytes in vitiligo. On the other hand, reduced concentrations of IL-10, a potent regulator of anti-inflammatory immune responses was observed in vitiligo patients (Singh et al., 2012). An increased IL-10 concentration with an increase in the IFNG levels exhibited a positive correlation with disease duration as reported in vitiligo patients (Ala et al., 2015). IL-4, an immunomodulatory cytokine, stimulates B-cell proliferation and T cell development that leads to the elevation of baseline IgE levels inducing inflammation (Del Prete et al., 1988). Polymorphisms in the IL4 gene are known to increase its expression increasing the IgE levels thereby implicating its role in autoimmunity mediated vitiligo susceptibility (Imran et al., 2012). CTLA4 expressed by Tregs is a negative regulator of T-cell function and foster tolerance to self-antigens. Decreased levels of CTLA4 mRNA and deregulated CTLA4 expression

due to genetic variations have been found in vitiligo patients (Dwivedi et al., 2011) suggesting its involvement in susceptibility to vitiligo. Also, upregulated CD44 expression in response to naive T cell proliferation as induced by autoimmune melanocyte destruction concomitantly increases T cell development implicating the complex regulation of self-reactive T cells in vitiligo (Byrne et al., 2014).

Increased mRNA levels of IL1B increases SOD levels leading to increased H_2O_2 production as observed in vitiligo patients. Genetic variability in IL1B resulting in altered IL1B transcript levels might be associated with elevated NPY levels in patients with vitiligo whose synthesis is governed by IL1B (Laddha et al., 2014). Increased NPY levels lead to epidermal and dermal hypoxia which might potentiate melanocyte death in vitiligo (Tu et al., 2001). Alterations in CAT have been reported to result in the reduction of the catalase enzyme activity and consequently evoke excess H_2O_2 accumulation in the entire epidermis of vitiligo patients (Casp et al., 2002). Although the genetic mechanisms of estrogen in increased pigment cell activity are not largely known yet, ESR1 expression on human melanocytes has been demonstrated to have specific actions in human pigmentation (Im et al., 2002). Also, genetic variation in ESR1 gene has been reported to show its association with vitiligo (Jin et al., 2004). Additionally, GSTP1 is broadly expressed in defense against oxidative stress wherein they detoxify a variety of electrophilic compounds generated by ROS-induced damaged cells (Nebert and Vasiliou, 2004). Altered GSTP1 expression fails to protect cells against chemical toxicity and stress contributing to melanocyte death in vitiligo patients (Dušinská et al., 2001; Liu et al., 2009).

Effector functions of IL2RA and GZMB in the target cell killing by cytotoxic T cells (CTLs) and NK cells activation-induced cell death terminate immune responses and mediate melanocyte killing in vitiligo (Spritz, 2010). GZMB also have a role to play in cleaving melanocyte proteins that constitute vitiligo auto-antigens activating auto-antigens that initiate and propagate autoimmunity directed against melanocytes (Darrah and Rosen, 2010). FOXP3, the master regulator of Treg cells, have a vital role in maintaining immune balance and its alteration triggers autoimmune diseases including vitiligo (Jahan et al., 2015). TNF down-regulates MITF affecting melanocyte development and proliferation, and ultimately affecting melanogenesis. Also, TNF-α downregulates MSHR binding activity and reduces MC1-R expression, both of which are known inducers of melanogenesis (Camara-Lemarroy et al., 2013). TRAIL, a TNF-family death receptor, activates caspases and cleaves melanocyte proteins and also promotes dendritic cell-mediated melanocyte death eliciting apoptosis of primary human melanocytes (Larribere et al., 2004). TNF, thus, acts as the central regulatory effector in the immunopathological mechanisms involved in vitiligo.

Among TLRs, TLR2 is fundamental for immune responses against mycobacterial infections, in sensing oxidative stress and cellular necrosis and, also in inducing apoptosis. (Petry and Gaspari,

2009). It also has the propensity to recognize a wide array of antigens evincing its instrumental role in the evolution of self-reactive diseases (Borrello et al., 2011). Altered expression and signaling due to TLR2 polymorphism have been proposed to be the reason for inadequate immune responses to viral or pathogenic antigens in vitiligo (Karaca et al., 2013; Traks et al., 2015).

The functional enrichment analysis result of the single large cluster consisting of 64 proteins demonstrated the vitiligo associated genes to be primarily involved in immune response regulation by cytokines and oxidative stress, and apoptotic processes. Oxidative stress in the melanocytes stimulates local inflammatory responses whereby it leads to the activation of innate immune processes as a result of which melanocyte-specific cytotoxic immune responses are evoked in vitiligo patients. Vitiliginous melanocytes show increased surface expression of HLA-A, a class I MHC receptor, which enables it to present multiple autoantigens to T cells destroying skin melanocytes (Hayashi et al., 2016). Also, increased expression levels of HLA class II molecules triggers an increased production of immunostimulatory cytokines that may act as an adjuvant during the presentation of autoantigens (Cavalli et al., 2016), tying together with HLA class I molecules in the development of autoimmunity in vitiligo patients. Also, alteration in the concentration of various pro-inflammatory and anti-inflammatory cytokines such as IL-10, IL-2, TNF, and IFNG has been associated with many autoimmune disorders (Singh et al., 2012).

Apart from exhibiting pathways associated with immune responses and autoimmunity, our pathway analysis results also consisted of pathways corresponding to infectious diseases, particularly viral infections. Several studies have implicated the etiopathogenesis of vitiligo to multiple viral infections as epidermal melanocytes are important targets of viruses (Duvic et al., 1987; Grimes et al., 1996). Also, viral infectious diseases, in most cases, impair the body's systemic immune response. This explains the reason why the pathways associated with infectious diseases were also observed to be significant in the results. In the protein-pathway network, TNF was found to be involved in 24 out of 30 significant pathways indicating it to be a prime regulator of vitiligo immunopathogenesis (Birol et al., 2006).

There is no definite cure available for vitiligo. The various treatment options available merely aim to improve skin appearance by repigmentation or stabilizing depigmentation without the assurance of reoccurrence or extension of depigmentation (Njoo et al., 1999). Our drug-target network analysis revealed novel potential drug candidates which could be explored for improved therapeutics for vitiligo. It is noteworthy that most of the drugs defined for the protein targets, even a few hub protein targets, are not indicated to be used for the treatment of vitiligo as reported in DrugBank. Monobenzone and Hydroquinone which targets TYR are the only drugs reported to be used in vitiligo with the latter being preferably used in

hyperpigmentation conditions. This indicates a pressing need to evaluate these drugs and perform investigational studies to identify new indications and elucidate the efficiency of these drugs for the treatment of vitiligo. This would lead to significant contributions in drug discovery complementing the existing drug pipelines, thereby, improving the quality of life in vitiligo patients. Additionally, some of the hub proteins are targeted by a single drug which can be further examined to contrive better effective drugs to enhance the success rate of treatments. Interacting drug partners of a particular drug targeting a particular protein might either directly target that protein or enhance the efficacy of the drug. With this concept, we constructed the drug-similarity network and found interacting drugs for those proteins which were previously shown to be targeted by a single drug in the drug-target network. These interacting drugs might function as an alternative to the native drug with enhanced efficacy.

Polymorphism in TLR2 (rs5743708) was found to be deleterious in our SNP analysis results indicating its potentiality to induce vitiligo. TLR2 was also found to be one of the hub protein targets for which no drug information is available yet. The R753Q mutation was identified to be located within the TIR domain, an intracellular signaling domain, which compromises the signaling capacity of TIR domain impairing MyD88-TLR2 assembly. This inactivates NF-κB signaling pathway which can invariably influence the regulation of inflammatory processes and can even impair melanogenesis suggesting its role in vitiligo pathogenesis (Karaca et al., 2013; Traks et al., 2015). Our PPI network of TLR2 shows its interacting proteins suggesting that an altered TLR2 might have an impact on its interaction with other proteins essential for many biological functions and signaling processes. This indicates the need to analyze the structural details of the protein and the effect of mutation on its structure and function, and carry out various experimental studies to discover new drugs targeting the mutated TLR2 protein associated with vitiligo.

CHAPTER 6

This chapter summarizes the present work and provides avenues and insights for future work.

CONCLUSION AND FUTURE PERSPECTIVES

A large-scale analysis and integration of miRNA-disease associations will offer a platform to investigate the patterns of the miRNAs and its associations with diseases. Molecular signature of miRNAs as reported in vitiligo patients suggests that these are actively involved and have a significant role in disease pathogenesis. Also, identifying the susceptible genes and their variants which drive the way to the onset of disease is of fundamental to unravel their contribution in disease induction. We identified vitiligo associated miRNAs and their targets, and susceptible genes, and carried out a comprehensive network analysis of these data which revealed the association of significant hub miRNAs and proteins with disease susceptibility. We validated their functional role and interpreted the biological activities and pathways in which they are involved. We also carried out SNP analysis and identified mutation in TLR2 (R753Q position) as deleterious. Our drug-target network and drug-similarity network unveiled novel molecular determinants and drug repositioning candidates for vitiligo. Our approach can provide an insight of the mechanisms of vitiligo development and progression, thereby, implicating its role in therapeutic as well as diagnostic applications. Furthermore, molecular dynamic simulation studies can provide a practical insight into the influence of SNPs on the TLR2 protein structure and function that ultimately initiates disease mechanisms. Further analysis can be carried out in inferring repositioning candidates based on the similarities between the prescribed (available) drugs and targets. Also, molecular docking studies can be performed to evaluate the efficacy of novel drugs for the identified unknown drug targets of vitiligo for improved therapeutic modalities.

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