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Original Article

Identification of key gene contributing to vitiligo by immune infiltration

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Abstract: Background: A deeper understanding of new prognostic and diagnostic biomarkers for vitiligo, an autoimmune disease, is needed. The purpose of this study is to identify the underlying long noncoding RNAs (lncRNAs) and immune infiltration related to the cause of vitiligo. Methods: The microarray data (GSE75819) were available to be downloaded from NCBI-GEO. Eight hub genes were identified from the Protein-protein interaction (PPI) network by the dissection of differentially expressed genes (DEG), Kyoto Gene and Genomic Encyclopedia (KEGG) expansion pathway, and Gene Ontology (GO). Further analysis based on the immune infiltration as well as the correlation between DEGs and immune cells was performed. Our conclusions were verified by using the GSE534 eventually. Results: According to our analysis, we obtained a total of 666 DEGs and 8 hub genes that include ECT2, CCT8, VRK1, UQCRH, EBNA1BP2, CRY2, IFIH1, and BCCIP, which may play an important role in vitiligo. Moreover, the immune infiltration profiles varied significantly between normal and vitiligo tissues. Compared with normal tissues, vitiligo tissues contained a greater proportion of mast cells ($P<0.05$). The analysis revealed that T cells regulatory (Tregs) have a negative correlation with the VRK1 expression ($R=-0:77$, $P<0.001$), whereas the mast cells resting have a positive correlation with the VRK1 expression ($R=0:72$, $P<0.001$) in vitiligo. Conclusion: The gene expression profile of vitiligo was realized by a bioinformatics method. The expressions of 8 hub genes and 22 immune cells were found, as the same as CRY2 and VRK1 have a special correlation with immune cells, which may be a significant cause of the pathogenesis of vitiligo. This provides a new idea for the diagnosis and treatment of vitiligo.

Keywords: Vitiligo, RNA, CIBERSORT, computational biology

Introduction

Vitiligo, an autoimmune skin disease caused by the loss of melanocytes, is a largely unexplained disease with a prevalence of 1% [1]. The complex interaction between non-immune and immune factors in vitiligo is the key to the pathogenesis of vitiligo. In addition to melanocytes, the involvement of keratinocytes, fibroblasts, natural killer cells and congenital lymphoid cells has been demonstrated. Vitiligo often recurs in the same area, indicating that vitiligo is closely related to memory T cells [2]. The mechanism of the formation of the vitiligo is due to internal abnormalities of keratinocytes and melanocytes. CD8 T cells play a major role in the loss of melanocytes in vitiligo through Damage-Associated Molecular Patterns (DAMPS) and adaptive immunity [3]. Clinically, vitiligo is characterized by the loss of melanocytes in the skin or hair leading to the development of the disease [4]. Topical glucocorticoids (TCS) have been used in the treatment of vitiligo through their immunomodulatory and anti-inflammatory effects. TCS and topical calcineurin inhibitors (TCI) are currently the most widely used therapies for vitiligo [5]. There have some combination treatments such as the combination of topical fluticasone propionate and UVA which was more effective than UVA [6]. At present, the results of the phase 2 trial of topical Ruxolitinib, a Janus-kinase inhibi-

tor, through the immune pathway against vitiligo have been published, which may mark the beginning of a new era for vitiligo therapy [7]. However, the underlying mechanism and effective treatment of vitiligo are lacking. Immune infiltration is a new research method that can explore the content of relevant immune cells in the disease. There has been previous research in this area. We explore the mechanism of vitiligo formation regarding the correlation between immune infiltration and differential genes, so as to better provide clinical diagnosis and treatment methods.

Methods

Data sources

The dataset GSE75918 downloaded from GEO database [8], was analyzed by microarray technology so as to examine mRNA expression profiles (platform: GPL6884 Illumina HumanWG-6 v3.0 expression beadchip), which contained 15 lesional skin tissues and 15 non-lesional skin tissues from vitiligo patients.

Differential analysis

The data were download from GEO database and the corresponding analysis was performed using the linear models for microarray data (LIMMA) method [9] (version 3.42.0) which is part of the R base package (version 3.5.3). This method involved the use of base-2 logarithmic transformations, employed to normalize the skew distribution and then to normalize the quantile. As for the GSE75918 data, the platform GP6884 was selected to assess the human genome in order to get the expression levels of mRNAs [10]. The DELs and DEGs between patients with lesional skin tissues and those with non-lesional skin tissues were identified using the LIMMA method [9]. DEG were selected on the basis of the false discovery rates (FDR) <0.05 and statistical threshold of |logFc (fold change)| >1; meanwhile, three lncRNAs were selected with the statistical threshold for false discovery rates (FDR) <0.05 and the highest difference expression in database for subsequent analysis. Two-way hierarchical clustering was applied using heatmap model from R package (version: 1.0.12;) to present a heatmap of DEGs.

Protein-protein interaction network of DEGs and select essential gene

PPI networks were predicted to get gene pairs by searching tools for interacting genes (STRING;) (version 11.0) [11] and online database. Furthermore, the PPI network of DEGs was obtained using STRING database, and their interaction of the composite score >0.4 was statistically significant. Then the PPI network was built using software which is Cytoscape (version 3.7.2;) [12]. We used Cytoscape software in our process of constructing PPI networks, which is an origin platform with bioinformatics software for visualizing molecular interaction networks. The MCode (version 1.4.3) in Cytoscape is a plugin applied to cluster a given network based on topology to find a densely connected area [13]. Besides, the essential module was identified by MCODE toward PPI network created by Cytoscape. Finally, we can find the hub gene from networks when degree >10 was set as the criterion for the key gene selection.

Function enrichment analysis

A functional enrichment analysis of Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomics (KEGG) was performed through a bioinformatics tool DAVID, especially its database for annotations, to predict the biologic function of DEGs [14]. A $P<0.05$ was considered to indicate significance.

Immune infiltration by CIBERSORT analysis

The proportions of 22 types of immune cells were obtained using the CIBERSORT algorithm and based on a further analysis of the normalized gene expression data generated in previous steps.

Sample selection depended on whether the *p* value was <0.05, and then we calculated the percentage of each immune cell in the sample. Next, we analyzed the different immune infiltration levels of each immune cell between the two groups through the analytical function of Vioplot package in R version 3.6.0.

Analysis of immune cells and genes

The method chosen for examination of the relationship between the immune cells and gene

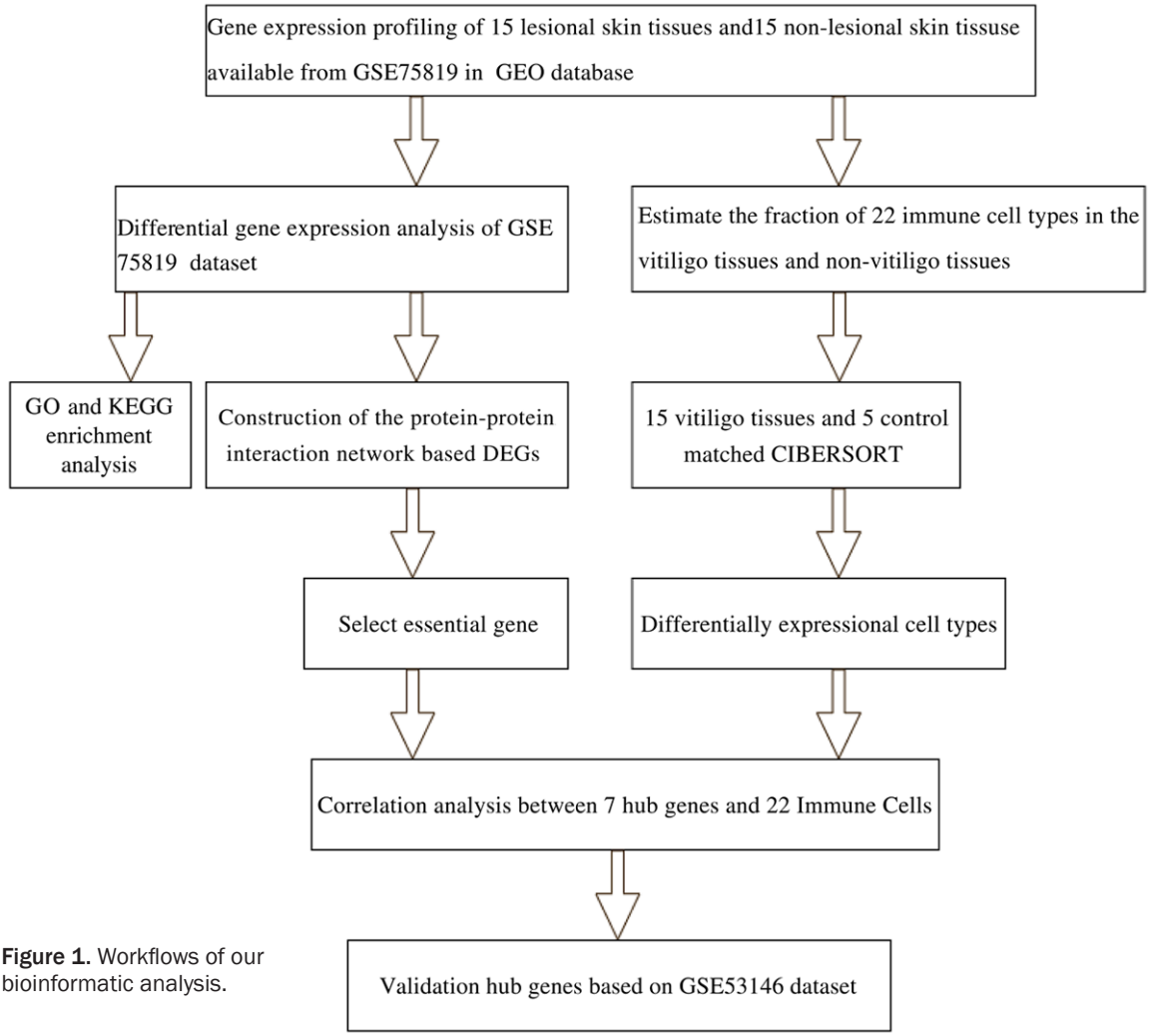

Figure 1. Workflows of our bioinformatic analysis.

expressions in samples was Pearson correlation test, in which a *p*-value <0.05 was considered as significant. Moreover, the moderate t-test was performed to identify differentially expressed genes. R version 3.6.0 was selected for dealing with the above data analysis.

Validation in the Gene Expression Omnibus dataset

Another dataset GSE53146 was sourced from the Gene Expression Omnibus (GEO), and the expression pattern of targeted DEmRNA was verified by the GSE53146 dataset containing 5 skin tissues of vitiligo patients and 5 controls.

Statistical analysis

All statistical analysis was conducted using R Statistical software (version 3.5.2). We explored the significance of fold changes observed in the microarray data based on t-test results. The relationships between immune cells and genes were compared by using Pearson correlation measurement. Unless otherwise indicated, a test result P<0.05 was considered significant.

Results

Our workflows are shown in **Figure 1**.

Identification of differentially expressed mRNAs

The first task was to obtain the microarray datasets GSE75918 from the public database GEO. We focused on the use of normalization as the method for drawing microarray results. According to the cut-off criteria (|logFc| >1 and Fdr <0.05), a total of 666 mRNAs (496 down-

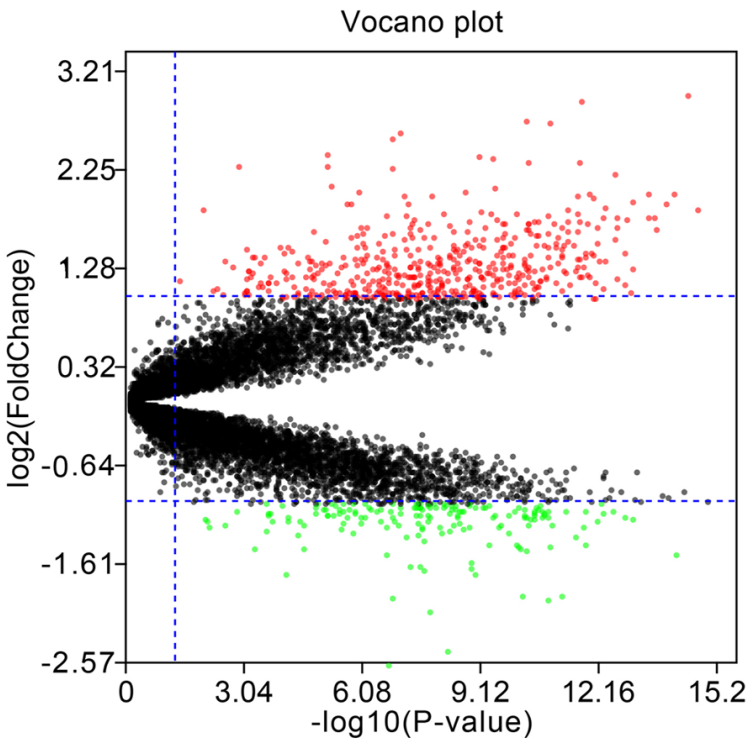

Figure 2. Volcano plot of all DEGs. Red means up-regulated and green means down-regulated DEGs.

regulated and 170 upregulated) were found in the gene chip GSE75819. A total landscape of gene expression in GSE75918 was offered in a volcano plot (**Figure 2**). The hierarchically clustered heat map indicated that the landscape of gene expression in GSE75819 was divided into a control group and a normal group (**Figure 3**).

DEG interaction network construction

Navigating the MCODE plugin in Cytoscape, we found 1712 edges and 396 nodes in the PPI from the network retrieved by the STRING database. Edges indicate interactions between DEGs and Nodes indicate DEGs. We then proceeded to select PPI network modules using the MCODE plugin so as to collect the most significant modules (**Figure 4**). A total of 8 genes with degrees ≥10 were identified as hub genes, including CCT8, BCCIP, CRY2, EBNA1BP2, IFIH1, UQCRH, VRK1, and ECT2. The signatures and functions belonging to 8 hub genes are listed in **Table 1**.

KEGG pathway analysis and GO term enrichment analysis

The 666 important DEGs were functionally classified using DAVID. The top 10 enrichment analyses for GO analysis are shown (**Figure 5**). For biological processes, he the functional significance of genes in translation (GO: 0006412), rRNA processing (GO: 0006-364), and SRP-dependent co-translational protein targeting to membrane (GO: 0006614) are shown in **Figure 5A**. For molecular function (MF) enrichment analysis, we showed that genes were mainly involved in Poly(A) RNA binding (GO: 0044822), Structural constituent of ribosomes (GO: 0003735) and Protein binding (GO: 0005515) as shown in **Figure 5B**. Moreover, by enrichment analysis of the cellular component (CC), genes that are mainly in the cytosol (GO: 0005829), ribosomes (GO: 0005840), and nucleolus (GO: 0005730), are shown in **Figure 5C**. KEGG pathway displayed that the DEGs were mostly in ribosomes (hsa03010), proteasomes (hsa03050), and oxidative phosphorylation (hsa00190). In these pathways the ribosome enrichment is predominant. The KEGG pathway enrichment analysis results are shown in **Figure 5D**.

Immune infiltration analyses

Using CIBERSORT algorithm, we first evaluated the differences of immune infiltration in 22 immune cell subsets in vitiligo and normal tissues. **Figure 6** shows the results summarized according to 5 normal controls and 15 patients with vitiligo. Compared to normal tissue, vitiligo tissues contained a higher proportion of mast cells resting and a lesser proportion of B cells memory, macrophages M2, dendritic cells resting, dendritic cells activated, and mast cells activated (**Figure 6**, $P<0.05$).

Analysis of immune cells and genes

We analyzed the correlation between immune cells and hub genes in normal and vitiligo samples (**Figure 7**). The results revealed that all other genes were correlated in a negative manner with Tregs, the same as in mast cells, except for the positive correlation between CRY2

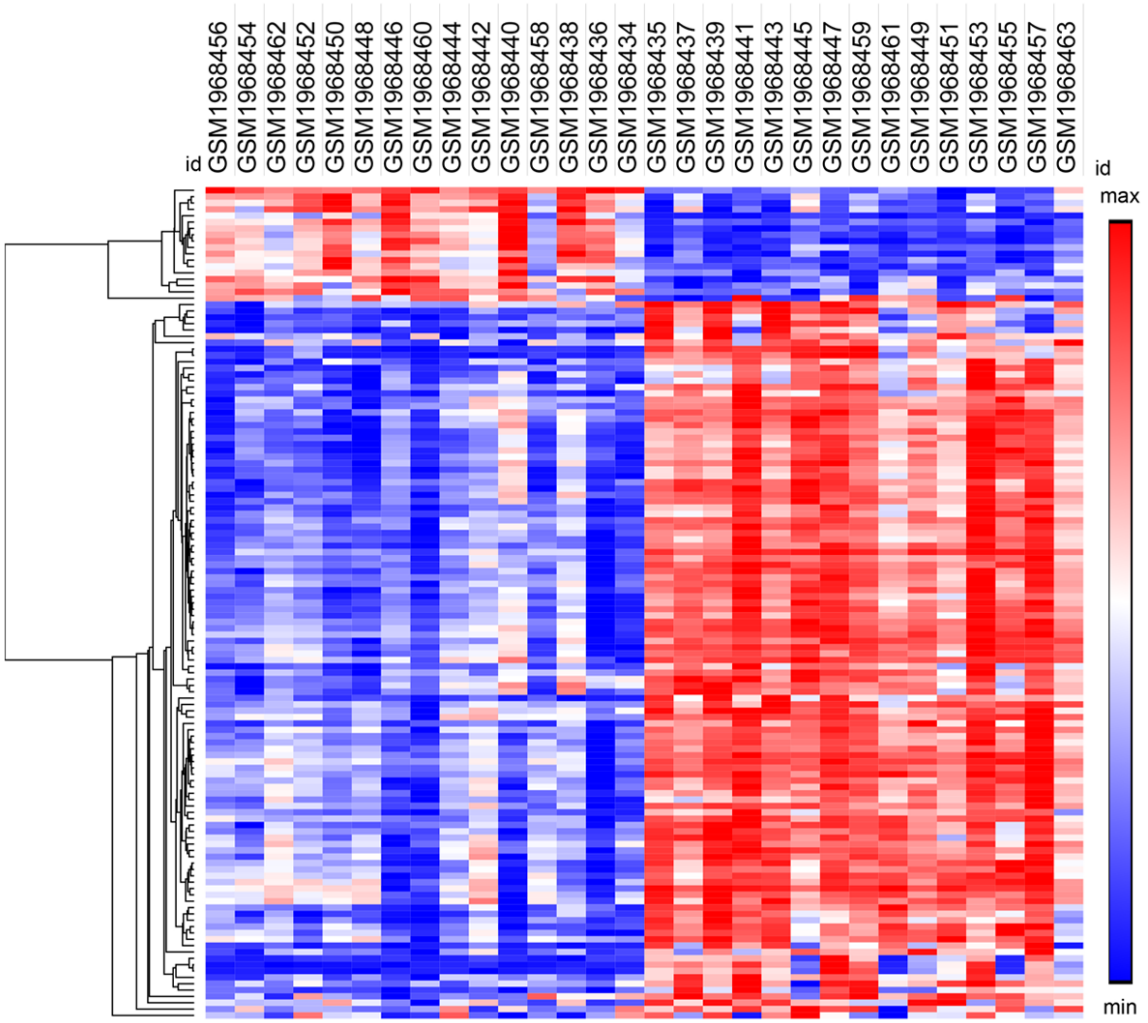

Figure 3. Heat map of the top 50 up-regulated genes and the top 50 down-regulated genes.

and T cells regulatory (Tregs) (R=0.56). Another finding is that only CRY2 was negatively correlated with mast cells resting (R=-0.55), but we observed a positive correlation between CRY2 and the other genes. The data showed a strong correlation between VRK1 and Tregs (R=-0.77, P<-0.001). There was also a strong correlation between VRK1 and mast cells resting (R=0:72, P<-0.001) (**Figure 8**).

Validation of the expression of DEmRNAs by GSE53416

The expression of UQCRH was validated according to GSE53416 (**Figure 9**). Based on our research, this result was generally consistent with those of our integrated analysis. The majority of selected key genes were up-regulated in the GSE53416 database such as CRY2, IFIH1, UQCRH and VRK1, except for CRY2 which was down-regulated, indicating that our results were convincing.

Discussion

The innovation of this experiment is that it is the first to use the method of immune infiltration to analyze the differential genes expressed in vitiligo based on bioinformatics, as well as its key analysis of the relationship between hub genes and immune cells which offers a new idea for its clinical treatment.

The mRNA enrichment showed that mRNA was mainly translation (BP), protein binding (MF), cytosol (CC), ribosome and oxidative phosphor-

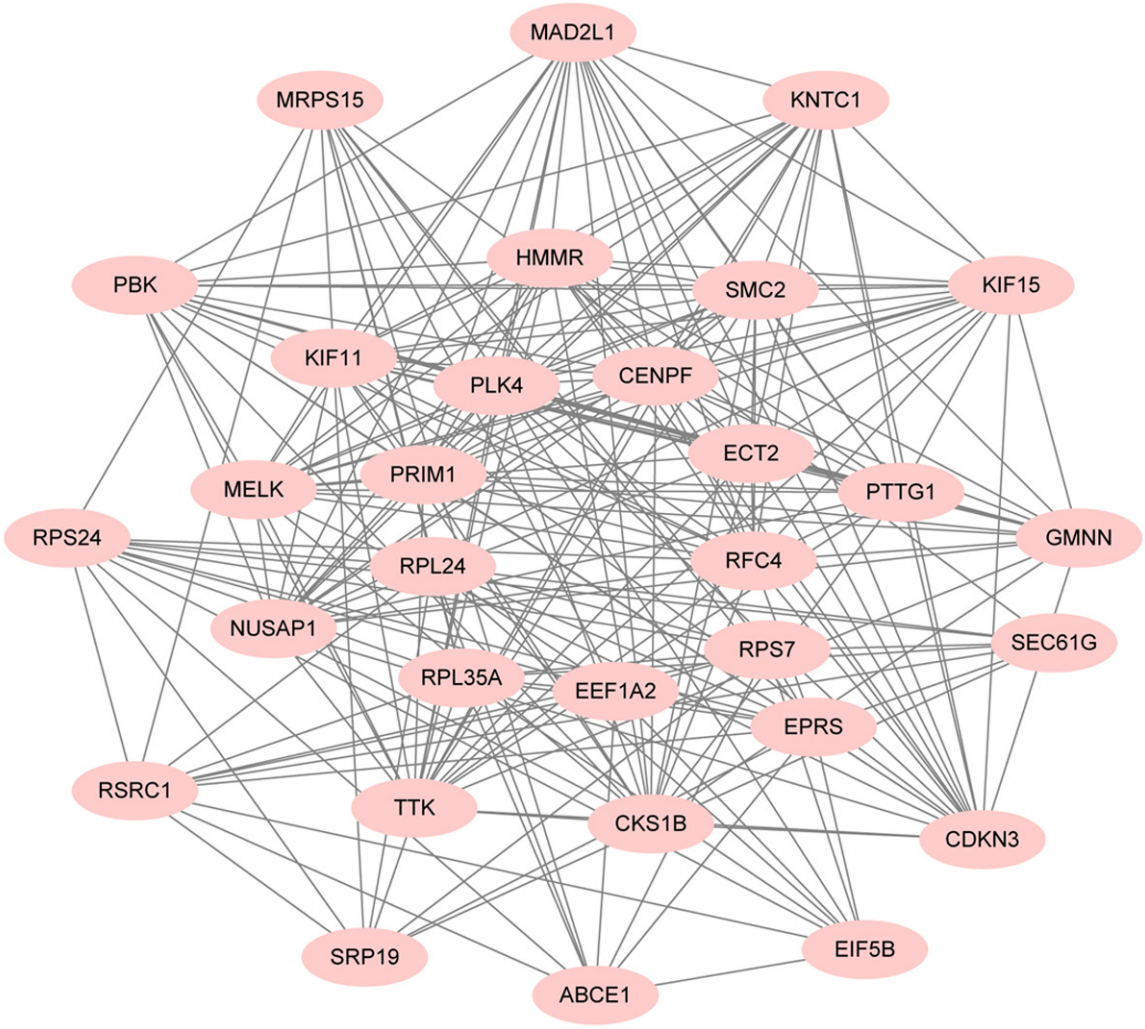

Figure 4. The most significant module was obtained from a PPI network.

Table 1. Introduction of eight keys genes

Gene symbol	Another name	Full name
ECT2	ARHGEF31	epithelial cell transforming 2
CCT8	Cctq; PRED71; D21S246; C21orf112	chaperonin containing TCP1 subunit 8
VRK1	PCH1; PCH1A	VRK serine/threonine kinase 1
UQCRH	QCR6; UQCR8	ubiquinol-cytochrome c reductase hinge protein
EBNA1BP2	P40; EBP2; NOBP	EBNA1 binding protein 2
CRY2	HCRY2; PHLL2	cryptochrome circadian regulator 2
IFIH1	AGS7; Hlcd; MDA5; MDA-5; RLR-2; IDDM19; SGMRT1	interferon induced with helicase C domain 1
BCCIP	TOK1; TOK-1	BRCA2 and CDKN1A interacting protein

ylation (KEGG). Sahoo et.al reported that the lower oxygen consumption rate and the increased production of reactive oxygen species in vitiligo cells seemed to be partly due to abnormal regulation of microRNA-211 and its target genes [15]. 26S proteasome in skin lesions of vitiligo patients decreased significantly [16]. Their findings are consistent with our enrichment analysis results. Furthermore, our analysis suggests that IFIH1 is most correlated with Tregs (R=-0.53). In previous studies, it has been confirmed that IFIH1 is closely relat-

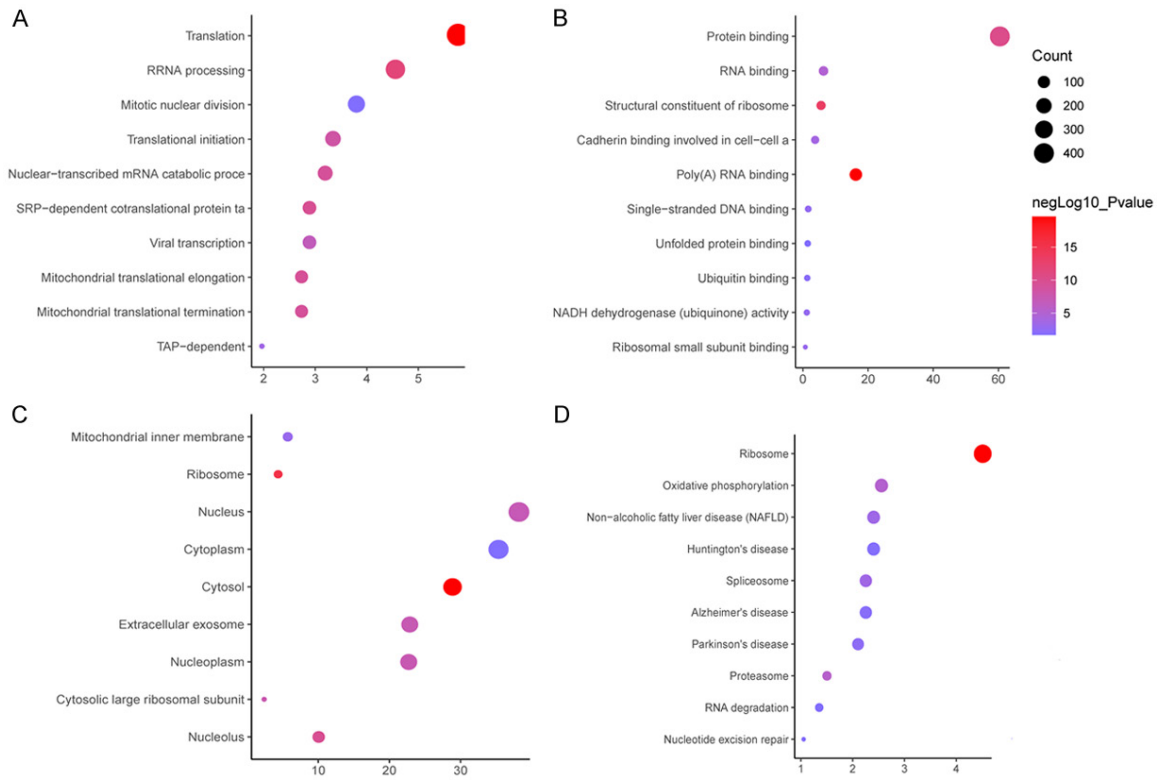

Figure 5. Enrichment analysis. A. Biological process. B. Molecular function. C. Cell component. D. KEGG pathway.

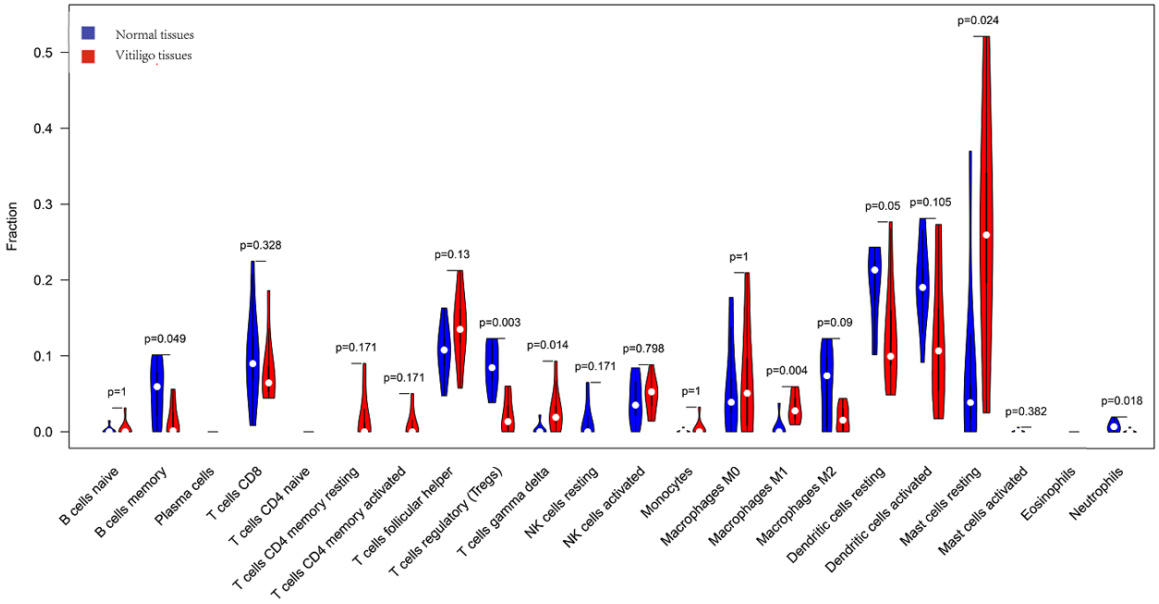

Figure 6. Immune infiltration: 22 types of infiltrated immune cells in groups.

ed to vitiligo [17], and Interferon type I plays an important role in the initiation of immune response in vitiligo [18]. Mutations in IFIH1 result in a series of neuroimmunologic features associated with enhanced interferon [19]. It can be inferred that IFIH1 is essential to the formation of vitiligo. It is speculated that IFIH1 can affect the immune system by Tregs.

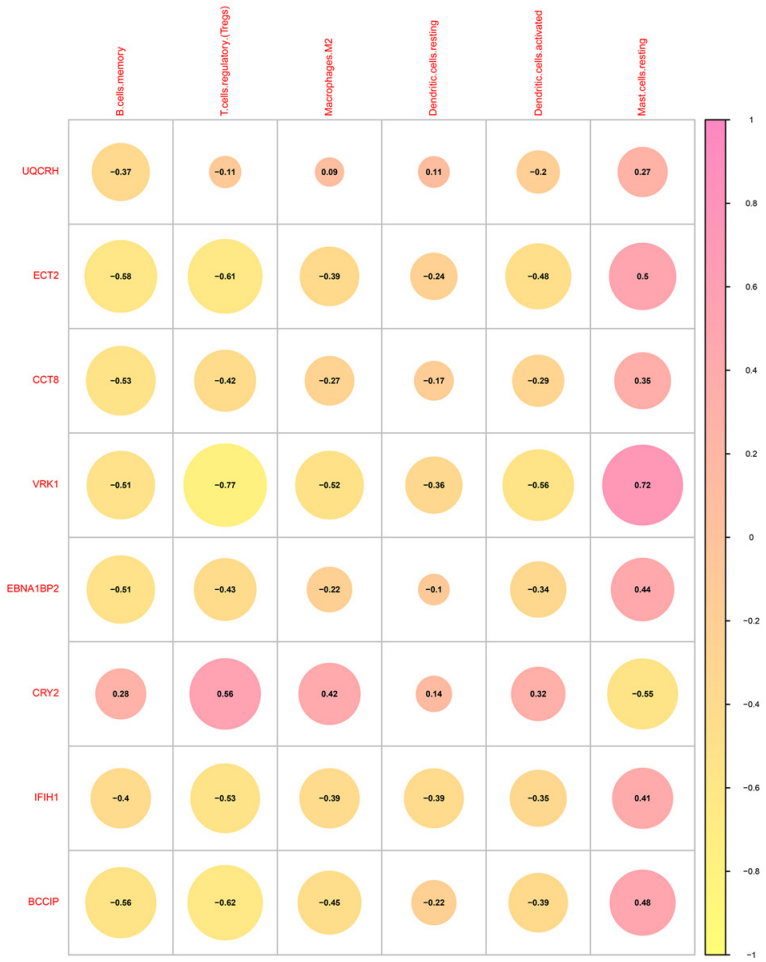

Figure 7. Correlation between immune cells and hub genes. Yellow represents low expression and pink represents high expression.

However, the direct relationship between Tregs and interferon requires further experiments to verify it. In the correlation analysis of key genes and immune cells, CRY2 was discovered to have a special correlation with immune cells. It was previously reported that the CRY2 gene exists in immune and endocrine cells and is expressed in human cells in a circadian manner [20]. Some endocrine markers play a role in vitiligo, including melatonin [21]. Melatonin regulates circadian rhythm and affects metabolic function [22]. We speculate that CRY2 affects the activity of immune cells by acting on the metabolic system. The correlation between VRK1 and immune cells was the highest, but there has been no study on this finding in the known reports, in previous studies, increased expression of p53 in vitiligo epidermis has been demonstrated [23], with increased oxidative DNA damage in mononuclear leukocytes in vitiligo [24]. Furthermore, we found that p53 and VRK1 formed a stable complex that induced DNA damage [25]; therefore, we speculate that VRK1 may affect vitiligo by affecting DNA damage.

Compared to previous studies, our study obtained 22 immune cell expression levels in vitiligo, identified 8 key genes, and predicted the possible mechanism between IFIH1, CRY2, VRK1, and immune cells. However, our research has some limitations. First, the CIBERSORT analysis may be limited based on inadequate genetic data that may deviate from the heterotypic interaction of cells, so CIBERSORT may show a lower estimation bias, and overestimate or underestimate some cell types. In addition, this study lacks relevant experiments for auxiliary verification, and in some instances the theoretical results may be different from the actual results. However, this study provides a novel research idea to explore the mechanism of vitiligo formation through the combination of health information analysis and immune infiltration. In future research, CRY2, VRK1, and related immune cells can be further analyzed to explore the specific mechanism of vitiligo formation, and provide a reference for clinical diagnosis and treatment.

Conclusion

This study provides further insights into the molecular mechanisms of vitiligo, leading to new targets for the development of specific biomarkers. To our knowledge, to date there has been no published research on immune infiltration in vitiligo. The key finding of this study is that vitiligo gene expression profiles are realized using a bioinformatic method. 8

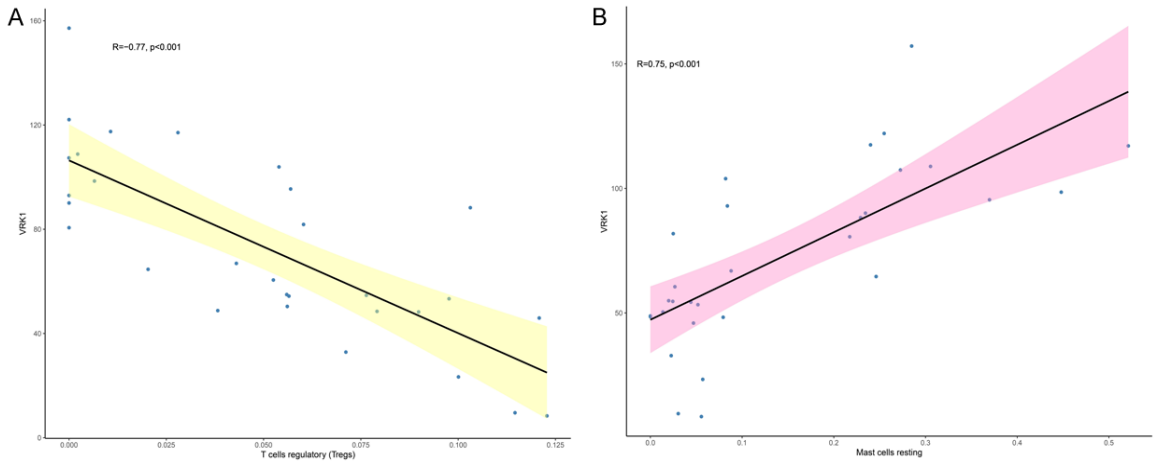

Figure 8. A. Correlation between CRY2 and T cells regulatory (R=0.56). B. Correlation between VRK1 and mast cells resting (R=-0.55).

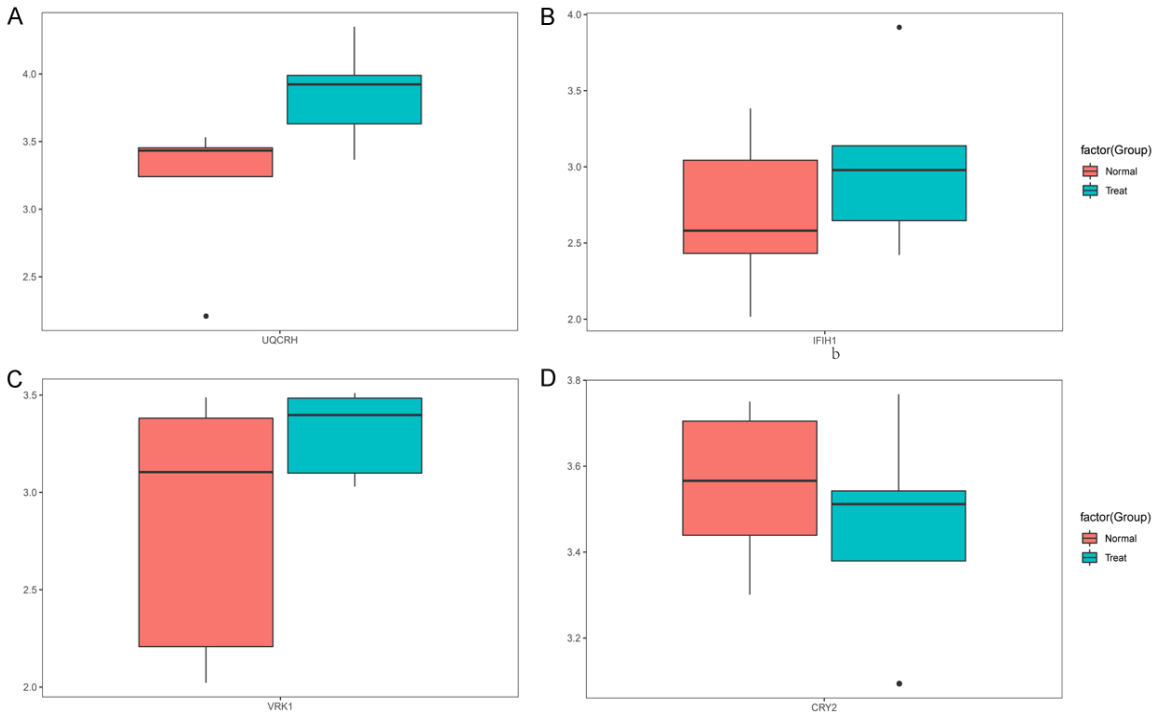

Figure 9. Validation of the expression levels based on GSE53146. UQCRH (A), IFIH1 (B), VRK1 (C) and CRY2 (D).

hub genes and the expression of 22 kinds of immune cells were found, that may play crucial part in vitiligo. Moreover, the correlation between key genes and immune cells was established, which may facilitate further understanding of the mechanism of vitiligo formation and provide useful biomarkers for the diagnosis and treatment of vitiligo patients.

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Disclosure of conflict of interest

None.

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