Integrative Analysis to Identify Diagnostic Biomarkers (HSP90AA1, VCAM1 and MX1) and Reveal Immune Cell Infiltration Changes in Patients with Vitiligo

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Article

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Abstract

Background

This current study applied bioinformatics analysis to reveal the potential role of immune-related genes and blood immune cell infiltration changes in vitiligo development.

Methods

The gene expression level was obtained from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were identified between blood samples from patients with segmental vitiligo (SV), non-segmental vitiligo (NSV) and healthy controls (CL), and the DEGS from two sets were used for further analysis respectively, including hub gene exploration, enrichment analysis, interaction network construction and engaged pathway analysis. Immune-related gene crosstalk analysis, gene-miRNA analysis, and AUC analysis were performed for further gene screening. Furthermore, immune cell infiltration analysis was also performed to reveal the change of systemic environment in patients with vitiligo.

Results

There were 161 DEGs in the comparison of SV and CL, 11 DEGs in NSV and CL. Functional enrichment analysis indicated that DEGS in NSV group mainly involved in responses to virus and type 1 interferon signaling pathway, while DEGS in SV group mainly involved in IL-17 signaling pathway and TNF signaling pathway. After PPI network construction, immune-related gene crosstalk analysis, and verification of diagnostic markers, totally 4 genes were obtained as diagnostic markers (VCAM1, HSP90AA1, TNF from SV group and MX1 from NSV group). Moreover, we found that the infiltrated percentage of resting memory CD4 T cells were both higher in blood samples from patients with segmental vitiligo and non-segmental vitiligo.

Conclusion

For the first time, VCAM1, HSP90AA1, and MX1 were found associated with vitiligo and had good diagnostic significance. Meanwhile, we found that vitiligo patients had more proportion of resting memory CD4 T cells infiltration in blood. These results may provide new research ideas for the pathogenesis and diagnosis of vitiligo.

Introduction

Vitiligo is an autoimmune disorder characterized by patchy loss of skin pigmentation with unclear etiology[1], ~30% of vitiligo patients are affected with at least one additional autoimmune disorder[2], which

means blood transcriptomics may provide new insights into disease mechanisms. In the occurrence and development of vitiligo, the immune cell infiltration and the abnormal expression of specific genes are closely related to the pathogenesis. Differences in gene expression may be related to the types of immune cells[3]. However, most studies have focused on changes in lesion skin, information on the systemic environment remains incomplete.

Two forms of the disease, segmental and nonsegmental vitiligo, are well recognized[4]. Because the disease course, prognosis, and treatment modalities are different between segmental and nonsegmental vitiligo, it is important to distinguish between these vitiligo forms at diagnosis[5]. However, the diagnosis of vitiligo mainly relies on clinical manifestations, which is extremely subjective. Reliable objective diagnostic basis can not only improve the clinical confirmation rate, but also increase the conviction of patients. Therefore, a better understanding of blood diagnostic biomarkers to distinguish vitiligo type is necessary indeed.

There is a growing interest in microarray platforms as a way to detect genetic alterations and to determine biomarkers for many diseases [6]. Several biomarkers and pathways have been implicated in the development of vitiligo in previous studies on microarray data[7,8], but still no studies focus on the potential blood diagnostic biomarkers of vitiligo. In this study, we compared blood samples from segmental vitiligo (SV) and non-segmental vitiligo (NSV) with healthy control by integrated bioinformatics methods. The DEGs from the two comparisons were used for further analysis, respectively. More importantly, we analyzed the immune cell populations of the different datasets. This study leads to an improved understanding of the etiology of segmental vitiligo as well as non-segmental vitiligo and effective therapeutic and diagnostic targets.

Materials And Methods

Dataset Collection

Gene expression datasets were collected from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo)[9].

Data processing and Differential Expression Analysis

Raw data were processed and analyzed using R (version 4.0.2). Firstly, the the RMA algorithm was used for background correction and data normalization and the result was shown through box plot. Then, the “limma R” package was used to identify DEGs[10] ($|Log2FC| > 1$, $p < 0.05$) between each group in the dataset, and a volcano map and heatmap of TOP 20 DEGs was drawn respectively.

Functional Correlation Analysis

Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA) on DEGs were performed using “clusterProfiler” package[11] “c2.cp.kegg.v7.0.symbols.gmt” was selected as the reference gene set. Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using GAGE[12] followed by visualization with the ggplot2 R package. Annotation of the genes and pathways was provided by the KEGG database (http://www.genome.jp/kegg). A false discovery rate (FDR) < 0.25 and $p < 0.05$ were considered significant enrichment.

Construction of the PPI network

To characterize the crucial DEGs in NSV and SV, we used an online tool STRING (https://string-db.org/) to construct PPI networks with a minimum required interaction score of 4[13]. For further analysis, Cytoscape software was used for the download interaction information. Significant genes were determined by the cytoHubba plugin as hub genes[14].

Gene-miRNA network construction

NetworkAnalyst (https://www.networkanalyst.ca/) is an online tool for target gene-miRNA network generation[15], which integrates the comprehensive experimentally validated miRNA-gene interaction data collected from miRTarBase and TarBase. Statistics of the total nodes, edges, and seed proteins were obtained from the mapping overview. The target gene-miRNA network was visualized using Cytoscape version 3.6.0 software. Vitiligo-related miRNAs (hsa-mir-145, hsa-mir-155, hsa-mir-196a-2, hsa-mir-99b, hsa-mir-125b, hsa-mir-199a-3p) were collected from the Human microRNA Disease Database (HMDD) v3.2 database[16]. Then, we compared the miRNA list from the above datasets and chose the overlapped genes.

Crosstalk analysis between hub genes and Immune-related genes

1793 immune-related genes were downloaded from ImmPort database (https://immport.niaid.nih.gov)[17] including genes related to cytokines, T-cell signaling pathway, B-cell signaling pathway, NK (natural killer) cell signaling pathway etc. Then we use Venn plot to determine the crosstalk between hub genes from two groups and immune-related genes.

ROC curve analysis

ROC curve analysis was performed on all genes selected to calculate the area under the ROC curve (AUC) to assess their discriminating power with respect to the 2 groups (NS and SE); statistical significance at ROC AUC ≥ 0.930 (or ROC AUC ≤ -0.930 for those variables was set.

Immune cell infiltration analysis

The CIBERSORT method with the LM22 gene signature from CIBERSORT website (https://cibersort.stanford.edu/)[18] was used to estimate the relative proportions of 22 immune cell subsets in order. The percentages of these types were visualized in a bar graph by using the ggplot2 package of R.

Results

Data preprocessing

After searching in Gene Expression Omnibus database with the inclusion criteria included: (1) patients with vitiligo; (2) blood samples, 2 datasets (GSE80009 and GSE90880) were obtained. For details, 12 samples (4 healthy controls, 4 segmental vitiligo, and 4 generalized vitiligo) from GSE80009, 14 samples (6 healthy controls and 8 non-segmental vitiligo) from GSE90880.

Identification of DEGS

We set totally 2 sets to identify DEGs respectively, including NSV group from GSE90880 (non-segmental vitiligo and healthy controls) and SV group (segmental vitiligo and healthy controls) from GSE80009. DEGs were identified with the setting of cutoff at FDR < 0.05 and |log2 (FC)| ≥1. As shown in the volcano plot, totally 11 DEGs from NSV group, 161 DEGs from SV group were obtained (Fig. 2C, D). The top 20 DEGs of the 2 sets are illustrated in heatmap plots (Fig. 2E, F). The DEGS of the two groups were analyzed separately in the subsequent.

Functional Correlation Analysis

Enriched GO terms were divided into three categories: BP, CC, and MF. As shown in Fig. 3B, the DEGs from the SV group were mainly enriched in the ‘positive regulation of vascular endothelial growth factor production’, ‘fever generation’, ‘astrocyte activation’ and ‘leukocyte chemotaxis’ in the BP group. In terms of MF, DEGs were most enriched in ‘peptide receptor activity’, ‘cytokine activity’, ‘cytokine receptor binding’ and ‘G protein-coupled peptide receptor activity’. KEGG analysis of DEGs revealed that they were mainly enriched in ‘cytokine-cytokine receptor interaction’, ‘TNF signaling pathway’, ‘IL-17 signaling pathway’ and ‘Rheumatoid arthritis’. Other results of DEGS from the NSV group were shown in Fig. 3.

GSEA results are also shown in Fig. 3. In the NSV group, the enriched pathways mainly involved cellular responses and neutrophil degranulation (Fig. 3E). While in the SV group, neutrophil degranulation as well as apoptosis modulation and signaling were mainly involved (Fig. 3F).

PPI Network Construction

As shown in Fig. 4A and C, totally 10 hub genes (TNF, IL1B, VWF, HSP90AA1, CAT, VCAM1, MMP1, IL1A, MMP3, TLR1) from SV groups and 9 hub genes (SERPING1, IFI44, ISG15, IFIT1, OASL, IFIT3, MX1, IFI44L, MX2) from NSV group were gained.

Construction of the Target Gene-miRNA Network

We totally obtained 14 genes (Fig. 5C), including 8 genes from SV group (TNF, IL1B, HSP90AA1, VCAM1, CAT, MMP1, IL1A, MMP3) and 6 genes from NSV group (IFI44, ISG15, IFIT1, OASL, IFIT3, MX1).

Crosstalk between hub genes and immune-related genes

As shown in Fig. 5D, 6 genes from SV group (HSP90AA1, IL1B, TNF, IL1A, VCAM1, and CAT) and 3 genes from NSV group (MX1, OASL and ISG15) were immune related.

Verification of Diagnostic Markers

As shown in Fig. 6, in NSV group (A), only MX1 met the requirement (AUC = 0.938); in SV group (B), AUC of VCAM1 and TNF was both 0.938, while HSP90AA1 met 1. When VCAM1 and TNF were fitted into one variable, the diagnostic efficiency was 1 (C), indicating that VCAM1 and TNF had a high diagnostic value. Conversely, none of these four diagnostic markers had an AUC value over 0.7 in the contrary group (D, E), which means those markers all had high diagnostic specificity in segmental vitiligo or non-segmental vitiligo.

Immune Cells Infiltration Results

The box plot of the immune cell infiltration difference showed that, compared with the normal control sample, patients with non-segmental vitiligo (Fig. 7A) had a higher ratio of CD8 T cells, resting NK cells and resting memory CD4 T cells, but a lower ratio of naive CD4 T cells and neutrophils. In SV group (Fig. 7B), the ratio of resting memory CD4 T cells, naive CD4 T cell and Neutrophils were higher, while CD8 + T cells was lower.

Discussion

In this study, we identified 4 highly specific diagnostic markers of blood for vitiligo for the first time, 3 for segmental vitiligo (VCAM1, TNF And HSP90AA1) and 1 for non-segmental vitiligo (MX1), and all four for immune-related genes. Among them, HSP90AA1 was upregulated, VCAM1, TNF and MX1 were downregulated. TNF, tumor necrosis factor, plays important roles in mediating the immune system and inflammatory systems[19]. TNF-α was reported increased in both vitiligo-involved skin sites and patients' serum in previous studies[20,21], while VCAM1, HSP90AA1 as well as MX1 are the first to kick out a possible association with vitiligo development. VCAM1 (Vascular cell adhesion molecular-1), also widely known as CD106, is a member of the immunoglobulin superfamily of proteins[22]. VCAM1 is an adhesion molecule which mediates the adhesion of leukocytes to the endothelium[23]. TNF-α could upregulate the expression of VCAM1, which led to leukocytes recruiting into inflammation site[24]. HSPs are prime candidates to connect stress and autoimmune responses to the skin. In particular, HSPs 60, 70, and 90 have been implicated in immune cell activation. Among them, only HSP-70 was found related to the immunological mechanism involved in the pathogenesis of vitiligo[25]. Newly formed HSP90AA1 can be secreted into the extracellular environment and can also enter the nucleus to stimulate the formation of immune memory[26]. Combined with previous studies, we speculate that HSP90AA1 may also be involved in the immune mechanism of vitiligo. Myxovirus Resistance 1 (MX1, also known as MxA) is the main mediator of IFN-based antiviral reactions, and MX1 is strongly affected by type 1 IFN[27]. Mx1 can cause hindrance in replication of RNA and DNA viruses[28]. In fact, several studies have linked the pathogenesis of vitiligo to the infection of a variety of viruses, such as cytomegalovirus, hepatitis virus, and HIV[29], but

the exact mechanism of viral infection leading to vitiligo has not been fully elucidated. MX1 may provide a new research target for vitiligo development.

In addition, we analyzed the changes of immune cell infiltration in the blood of patients with non-segmental vitiligo as well as segmental vitiligo. We found for the first time that the ratio of resting memory CD4 T cell was increased both in SV and NSV patients. As reported, the attribution of memory CD4 T cells included increased cytokine production, regulation of innate immune cell functions, mobilization of immune cells to sites of infection, providing B cell help, and enhancing cytotoxic T cell responses[30]. Patients with MS and psoriasis show increased numbers of memory CD4 T cell as compared with healthy individuals, suggesting that memory CD4 T cell are critical mediators of autoimmune disease. Actually, until now, T cell memory has been more extensively investigated in the context of infectious diseases and its role in autoimmune diseases is not fully elucidated[31], specially in vitiligo, which may provide a new insight into the pathogenesis and therapeutic targets of vitiligo.

In conclusion, we found for the first time that VCAM1, HSP90AA1 and MX1 are associated with vitiligo and have good diagnostic significance. Meanwhile, we found that vitiligo patients had more proportion of resting memory CD4 T cell infiltration in blood. These results may provide new research ideas for the pathogenesis and diagnosis of vitiligo.

Declarations

Author Contributions

Yibin Fan: study concept and design. Xiaoxia Ding, Youming Huang and Danfeng Xu: data analysis and manuscript drafting. All authors reviewed and approved the manuscript prior to its submission.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of Data and Materials

The datasets generated and analysed during the current study are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo).

1793 immune-related genes were downloaded from ImmPort database (https://immport.niaid.nih.gov).

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Figures

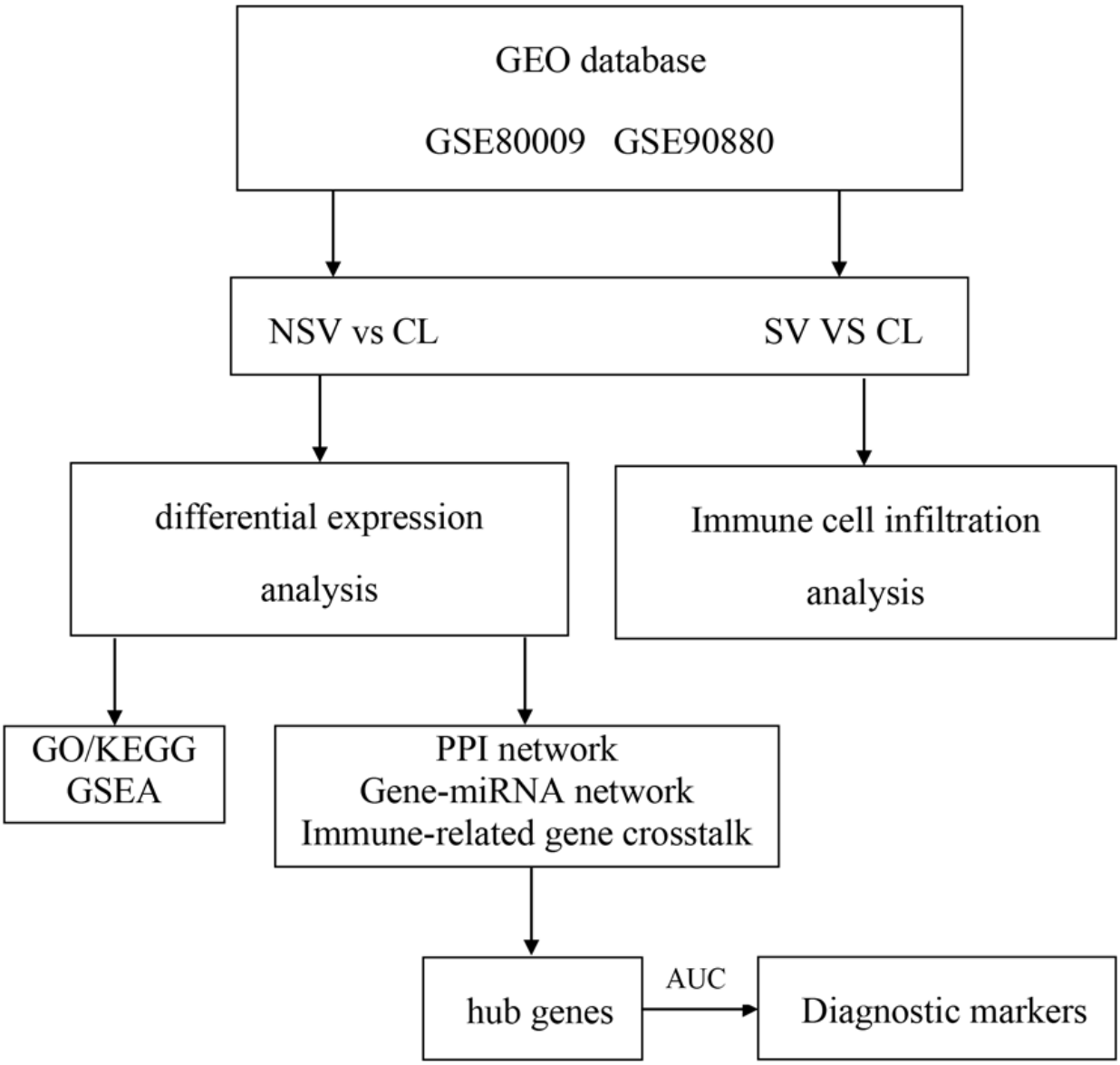

Figure 1

Flowchart of Methods

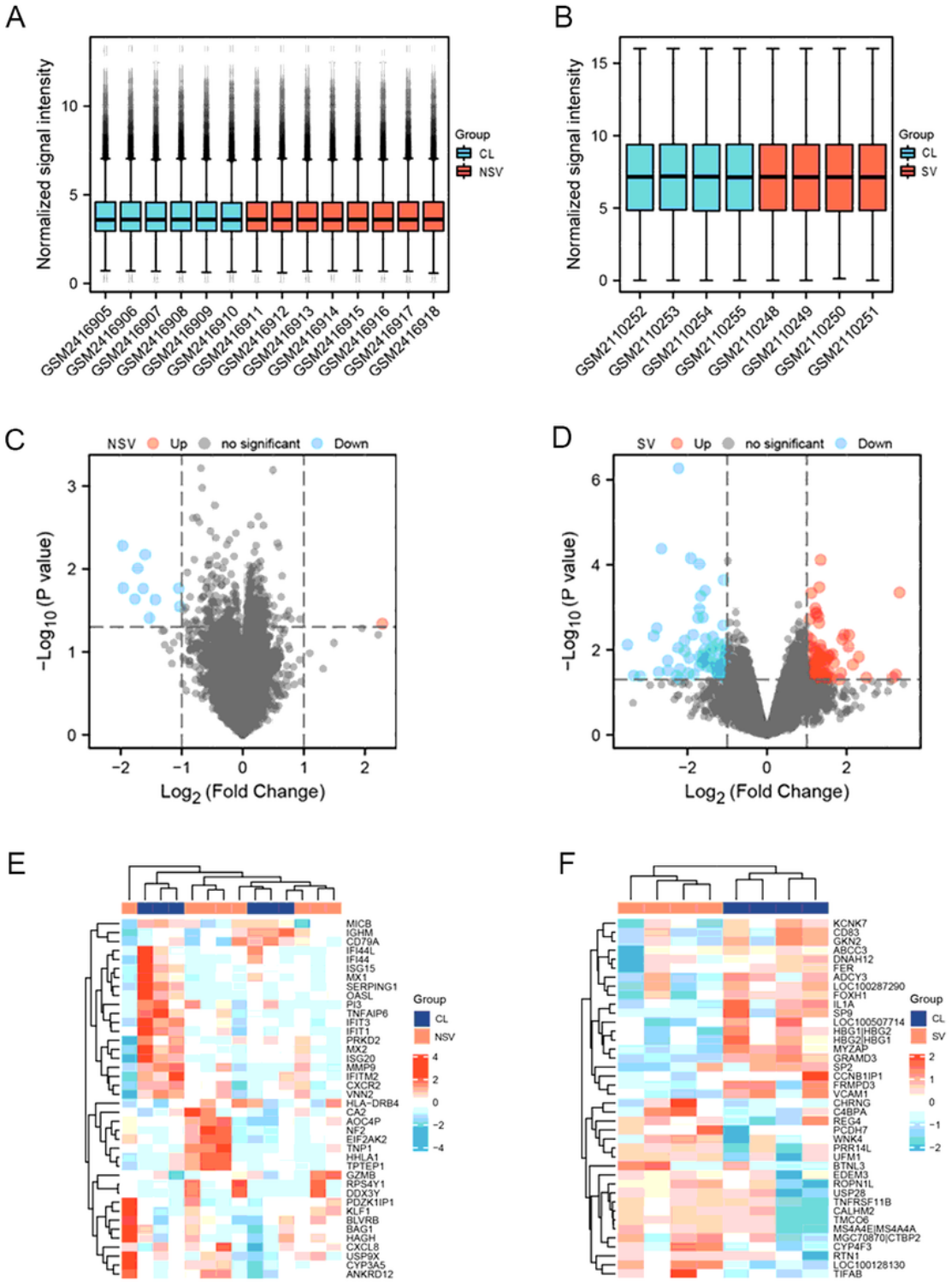

Figure 2

Normalization of microarray dataset and Identification of DEGS. A: Box plot after data standardization of NSV and CL group. B: Box plot after data standardization of SV and CL group. Volcano plots of DEGS from NSV group (C) and SV group (D). The red point in the plot represents the over-expressed mRNAs and the blue point indicates the down-expressed mRNAs with statistical significance. Heatmap of top 20 DEGs of NSV group (E) and SV group (F).

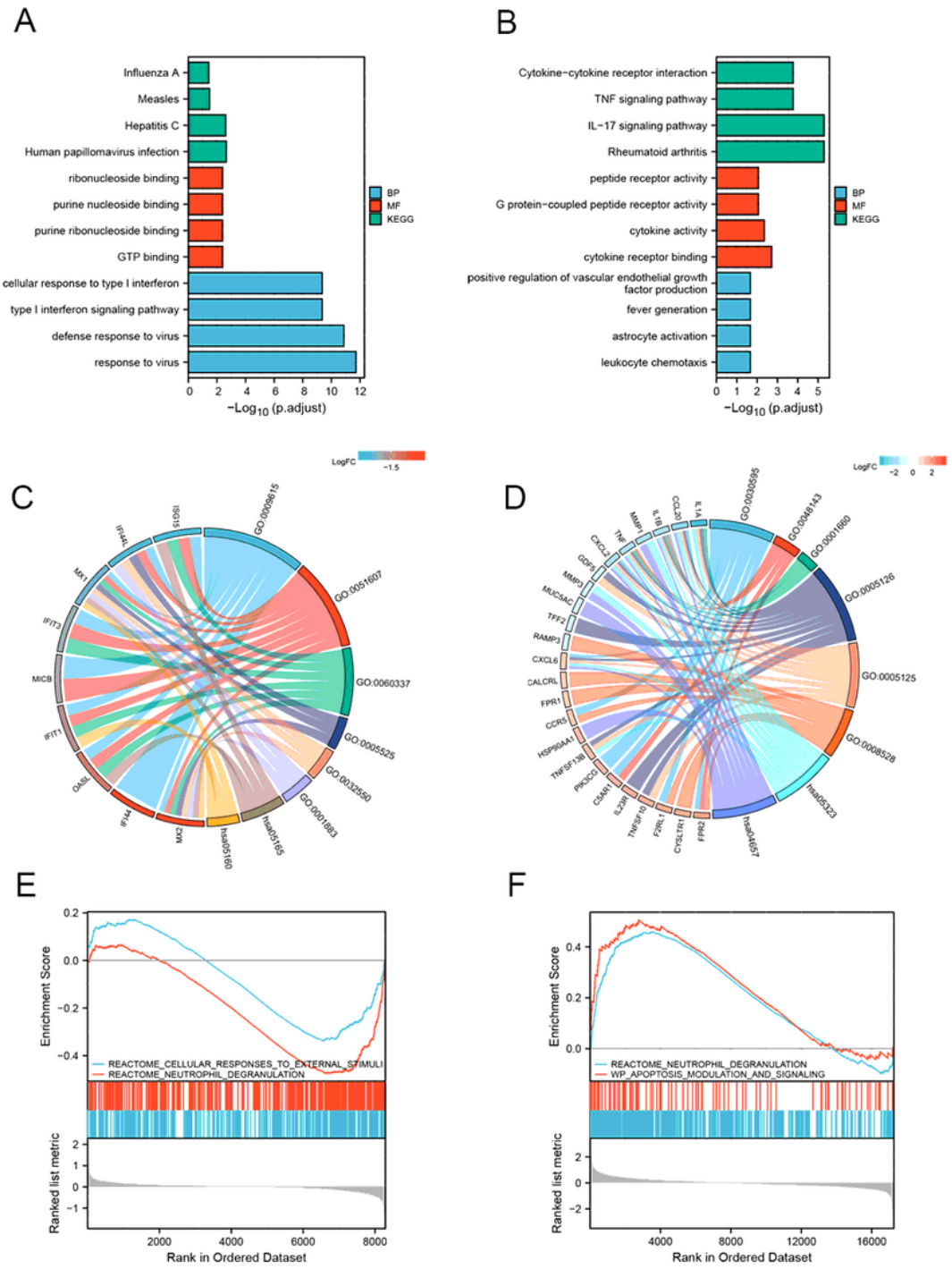

Figure 3

Enrichment analysis of DEGs. A and C: Enriched KEGG pathways and GO analysis in NSV group. B and D: Enriched KEGG pathways and GO analysis in SV group. E: GSEA results of NSV group; F: GSEA results of SV group; In the enrichment result, p <0.05 or FDR <0.05 is considered to be enriched to a meaningful pathway. BP: The biological process; CC: cellular component; MF: molecular function.

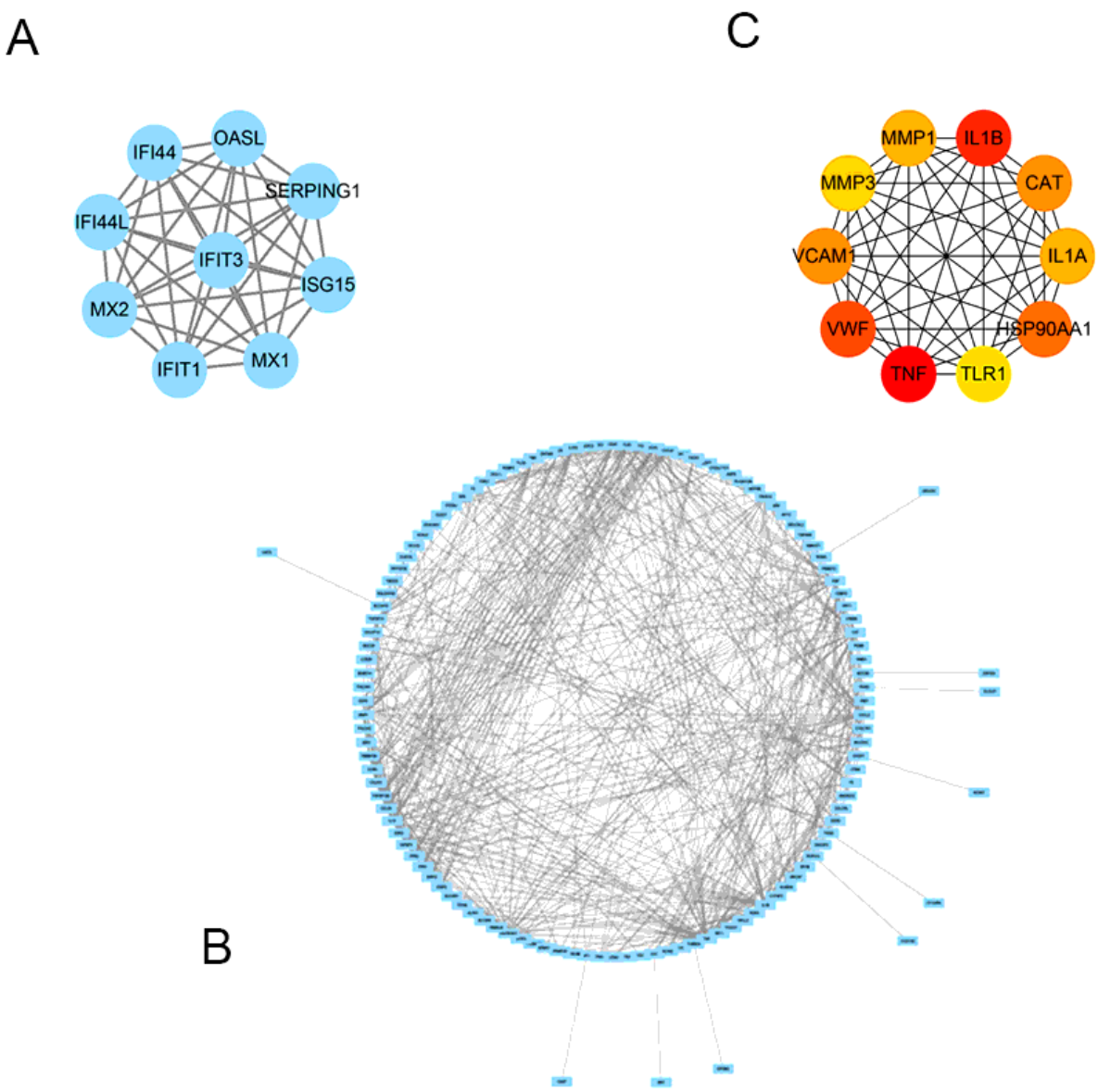

Figure 4

PPI network and interaction analysis. A: PPI network of DEGs in NSV group. B: PPI network of DEGs in SV group C: The top 10 hub genes determined by cytoHubba.

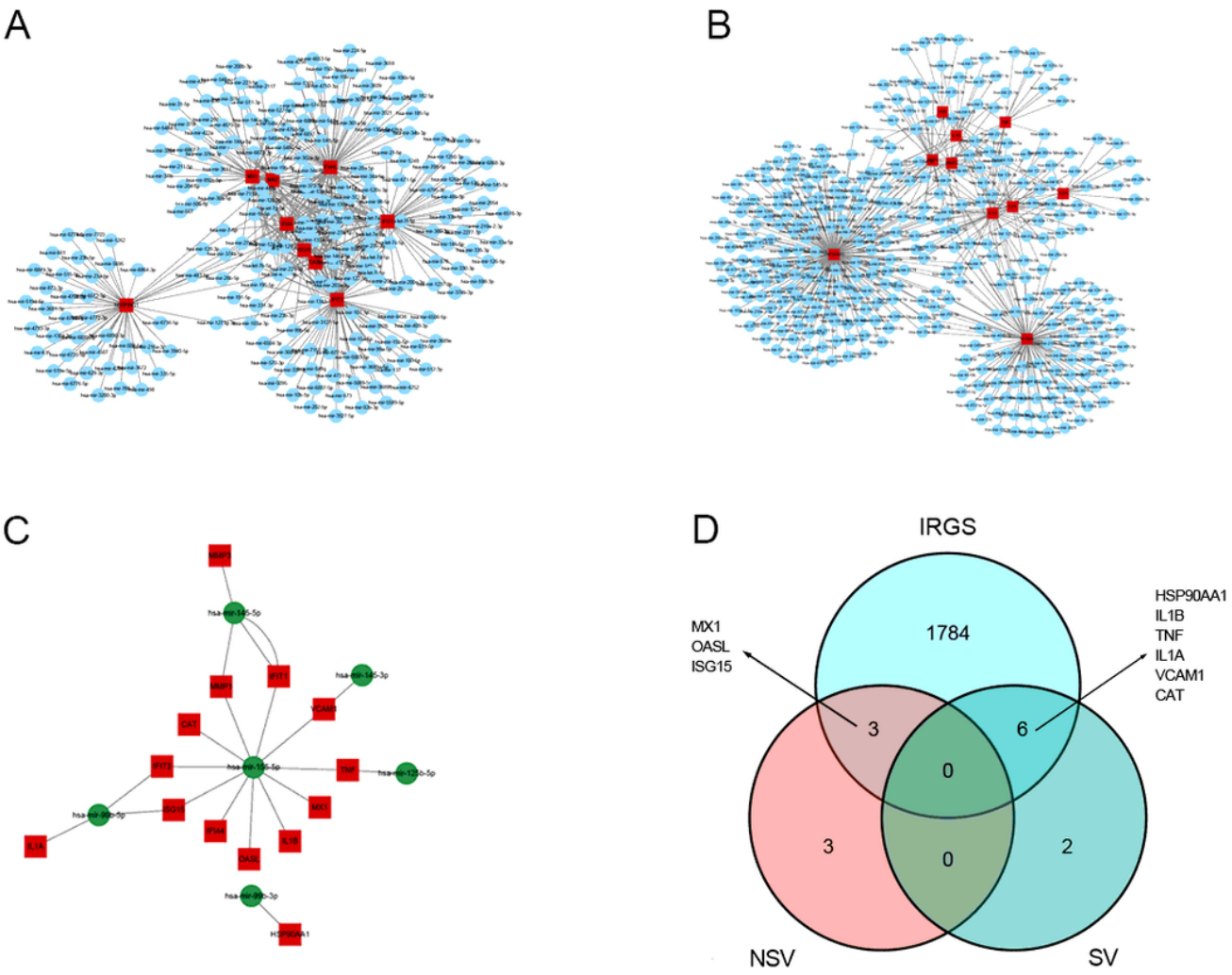

Figure 5

Gene-miRNA network construction. A: Gene-miRNA network of DEGS from NSV group; B: Gene-miRNA network of DEGS from SV group; C: hub genes and vitiligo-related miRNA network; D: Venn plot of immune-related genes and hub genes

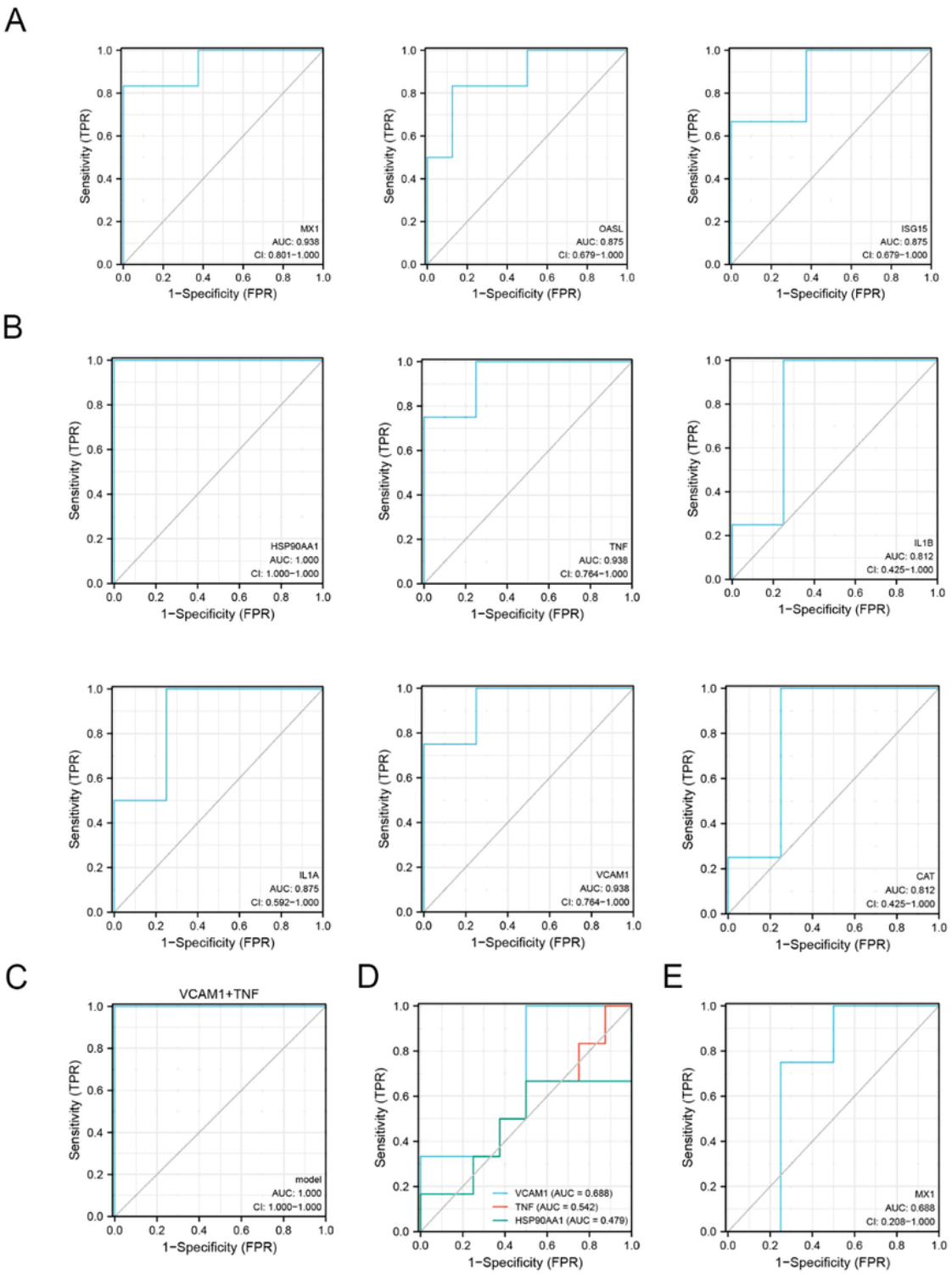

Figure 6

Diagnostic ROC curve. A: AUC analysis for hub genes from NSV group. B: AUC analysis for hub genes from SV group. C: Joint Metrics ROC curve of VCAM1 and TNF; D: ROC curve of MX1 in SV group; E: ROC curve of VCAM1, HSP90AA1 and TNF in NSV group

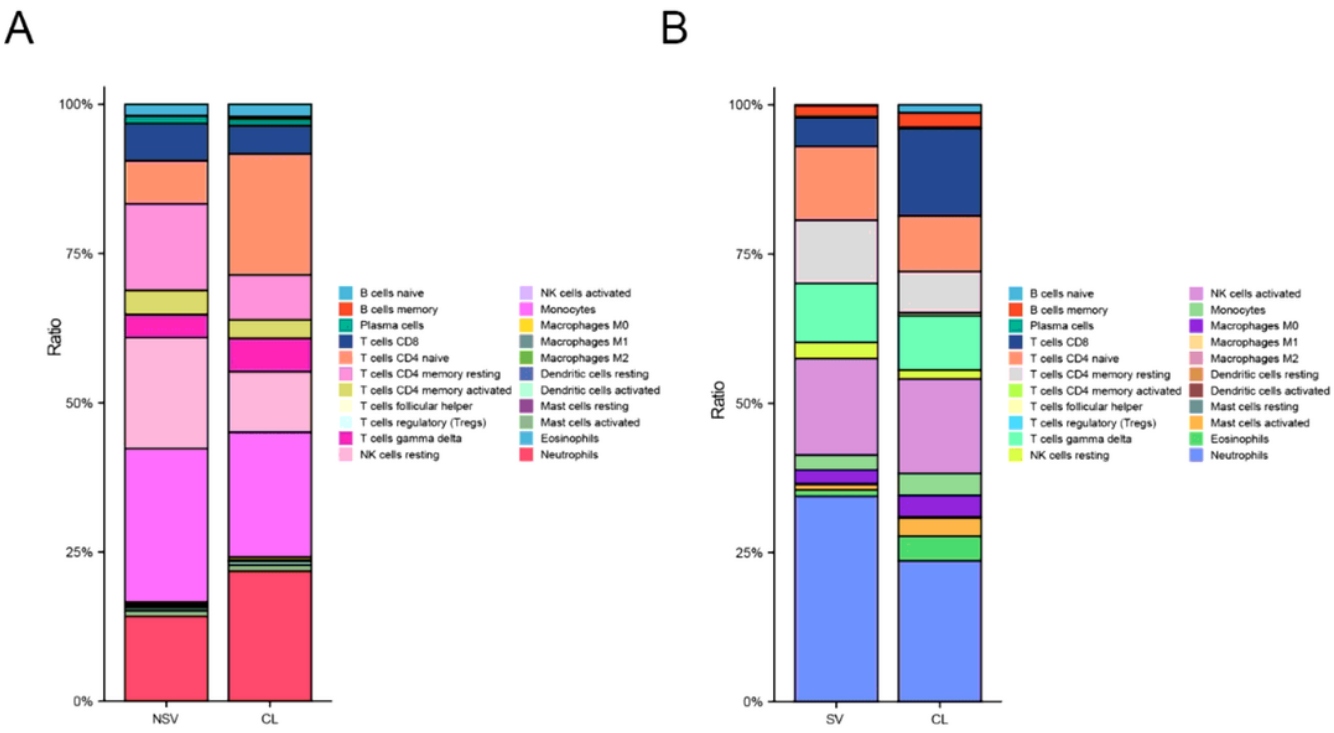

Figure 7

Immune cell infiltration analysis. A: Immune cell infiltration ratio in NSV and CL; B: Immune cell infiltration ratio in SV and CL