Immune Infiltration Pattern Associated with Diagnosis and Development in Benign Prostatic Hyperplasia

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Purpose: Benign prostatic hyperplasia (BPH) significantly reduces the quality of life. However, the biological mechanisms of BPH development remain largely unknown. We aimed to investigate the essential genomic and immunogenic features in BPH.

Materials and Methods: Transcriptome profiling and clinical data of BPH and normal prostate samples were acquired from GEO datasets. The discovery sets were composed of GSE119195, GSE7307, GSE101486, while the validation set was GSE132714. ESTIMATE and CIBERSORT were used to investigate the immunogenic features. Furthermore, transcriptional and weighted gene co-expression network analysis (WGCNA) was used for further analysis.

Results: BPH samples presented a higher immune score. Meanwhile, CIBERSORT deconvolution revealed that BPH exists significantly abundant M2 Macrophages, follicular T helper cells, resting mast cells, and fewer plasma cells, activated CD4+ memory T cells, and activated mast cells. WGCNA analysis also revealed significantly enriched immune-related modules in BPH. Transcriptomic analysis identified SOCS3, IL6, C3, IGF1, NOTCH1, and VCAN as key regulators of immunogenic phenotype in BPH. Moreover, we generated an immunological gene signature for BPH, which worked well in the validation cohort.

Conclusion: In our study, BPH samples exhibited a distinct immune infiltration pattern, represented by an immunological gene signature. This genomic-based assessment model reveals the potential transcriptomic patterns during BPH development.

Keywords: benign prostatic hyperplasia; transcriptomics; immune cell infiltration; diagnostic biomarker

INTRODUCTION

Benign prostatic hyperplasia (BPH) is the most common cause of bladder outlet obstruction and lower urinary tract symptoms (LUTS) in elderly males, which significantly reduces their quality of life.[1] The prevalence of BPH increases with age. Histologic BPH is found in approximately 50% men above 50 years old.[2] Although medical therapies including 5-alpha reductase inhibitors, alpha-blockers, and PDE5 inhibitors have been approved in BPH, a large number of patients still suffer from LUTS, particularly, urinary retention.[3,4] The histology of BPH is the overgrowth of stromal and epithelial cells, mainly in the transition zone of the prostate. The increase of cells is found in both the epithelial and stromal compartments. The pathophysiology of BPH was associated with androgen signaling, reactive stroma and inflammation.[1,2] Androgen signaling leads to activation of prostatic stroma signals, resulting in secretion of growth factors.[5-7] These factors result in epithelial proliferation and differentiation. Recently, BPH-associated prostate inflammation has also been proposed to be an important driver of BPH.[8-10] BPH-associated prostate inflammation may promote myofibroblast phenoconversion and prostatic fibrosis by the production of chemokines and growth factors, which may be enhanced by metabolic syndrome in aged murine.[11,12] However, the development and mechanism of immunogenic features and immune infiltration patterns during BPH development have not been determined yet.

Genomic technologies have been widely used in the studies of human diseases. In BPH, genomics technologies were used during recent twenty years ago.[13,14] In the present study, we aimed to investigate the essential genomic and immunogenic features in benign prostatic hyperplasia by transcriptomic analysis.

PATIENTS AND METHODS

Study design

The study workflow is presented in **Figure 1**. We col-

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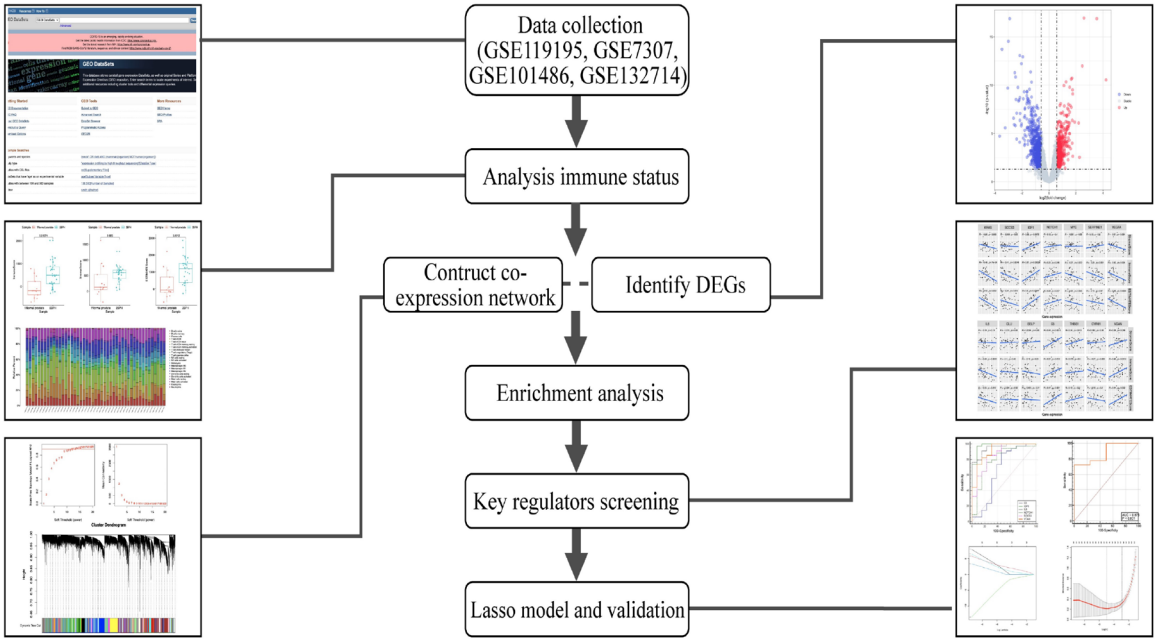

Figure 1. Flow chart of the study design.

lected microarray data as discovery set and validation set, respectively, from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) for available data of BPH. Immune status assessment was performed in the discovery set. Then, weighted gene co-expression network analysis (WGCNA), differentially expressed genes (DEGs) and protein-protein interaction (PPI) analysis were used to identify key regulators related to immune infiltration pattern. Finally, a diagnostic model was constructed by the Least Absolute Shrinkage and Selection Operator (LASSO).

Patients

We searched Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) for available data of BPH. At last, Transcriptome of 34 BPH samples and 13 normal prostate samples were collected from GEO datasets (GSE119195, GSE7307, GSE101486) as the discovery dataset. Batch effect was normalized by Combat function from "sva" package.[15] Another dataset (GSE132714) including 18 BPH samples and 4 normal prostate samples, was used as the validation dataset.

Immune microenvironment analysis

The ESTIMATE algorithm was used for quantification of immune and stromal scores.[16] The CIBERSORT analysis (https://cibersort.stanford.edu) by LM22 signatures with 1000 permutations was applied for evaluation of infiltrating immune cells.[17]

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a systematic biology method used to describe the pattern of gene correlation between BPH samples and normal prostate samples, as previously described.[18] Unsigned network and Pearson correlation were used in WGCNA analysis. The association between gene sets and phenotypes identifies potential biomarker genes which may be related in BPH development. WGCNA was performed using the "WGCNA" package in R software.

Functional and pathway analysis

Differential expression genes were determined by "Limma" package.(19) GO and KEGG enrichment analysis was conducted to explore pathway enrichment using metascape (http://metascape.org/). PCA was used to examine the clustering efficacy of the selected signatures.

Protein-protein interaction network construction and module analysis

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was applied for predicting the Protein-protein interaction (PPI) network and detecting the possible relationships (confidence score 0.4, maximum number of interactors = 0).

RESULTS

Different immunological status in BPH and normal prostate tissues

The flow chart of the study is presented in Figure 1. A discovery cohort containing 34 BPH and 13 normal prostate samples with available expression data and clinical information from GEO database was analyzed. Firstly, we calculated the immune score along with stromal score, in BPH and normal prostate tissues, by ESTIMATE algorithm. The immune score ($P < .001$), stromal score ($P = .025$) and ESTIMATE score ($P < .001$) were significantly elevated in BPH tissues (**Figure 2A**). The ESTIMATE results indicated a highly-enriched immunological status in BPH patients. Therefore, to investigate the change of immune infiltration during BPH development, we performed the CIBERSORT deconvolution to assess immune cell infiltration in tissue samples (**Figure 2B**). The landscape of immune infiltration in BPH has not been entirely explored yet, particularly the infiltration of different immune cells. Interestingly, we found that BPH samples

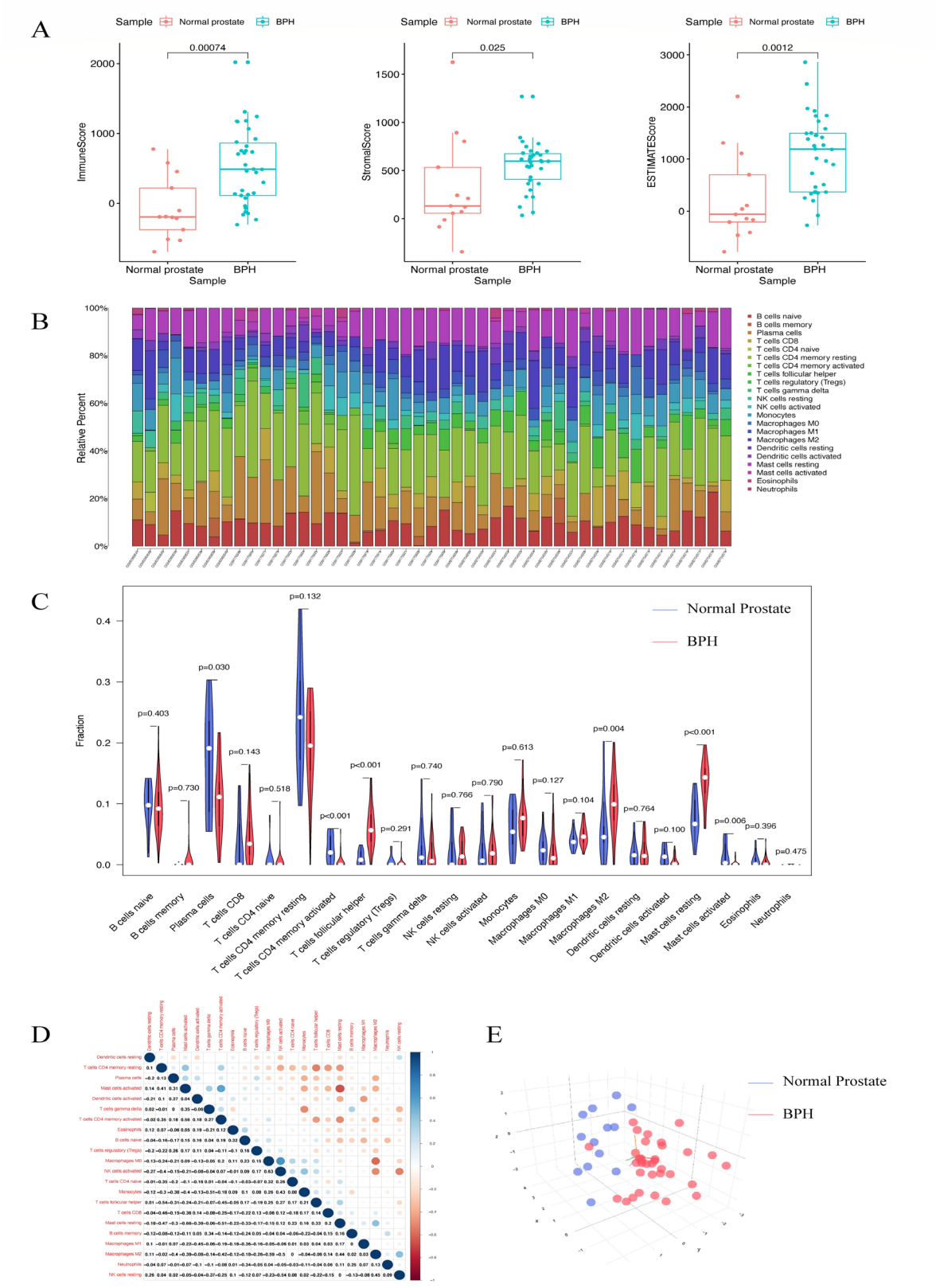

Figure 2. Different immunological status between BPH and normal prostate tissues. **(A)** ESTIMATE score, immune score and stromal score in BPH and normal prostate tissues. **(B)** Immune cell infiltration in BPH and normal prostate tissues according to CIBERSORT deconvolution. **(C)** Different immune cell infiltration between BPH and normal prostate tissues. **(D)** Correlation between immune cell infiltration. **(E)** Three-dimensional principal component analysis of BPH and normal prostate tissues according to immune cell infiltration.

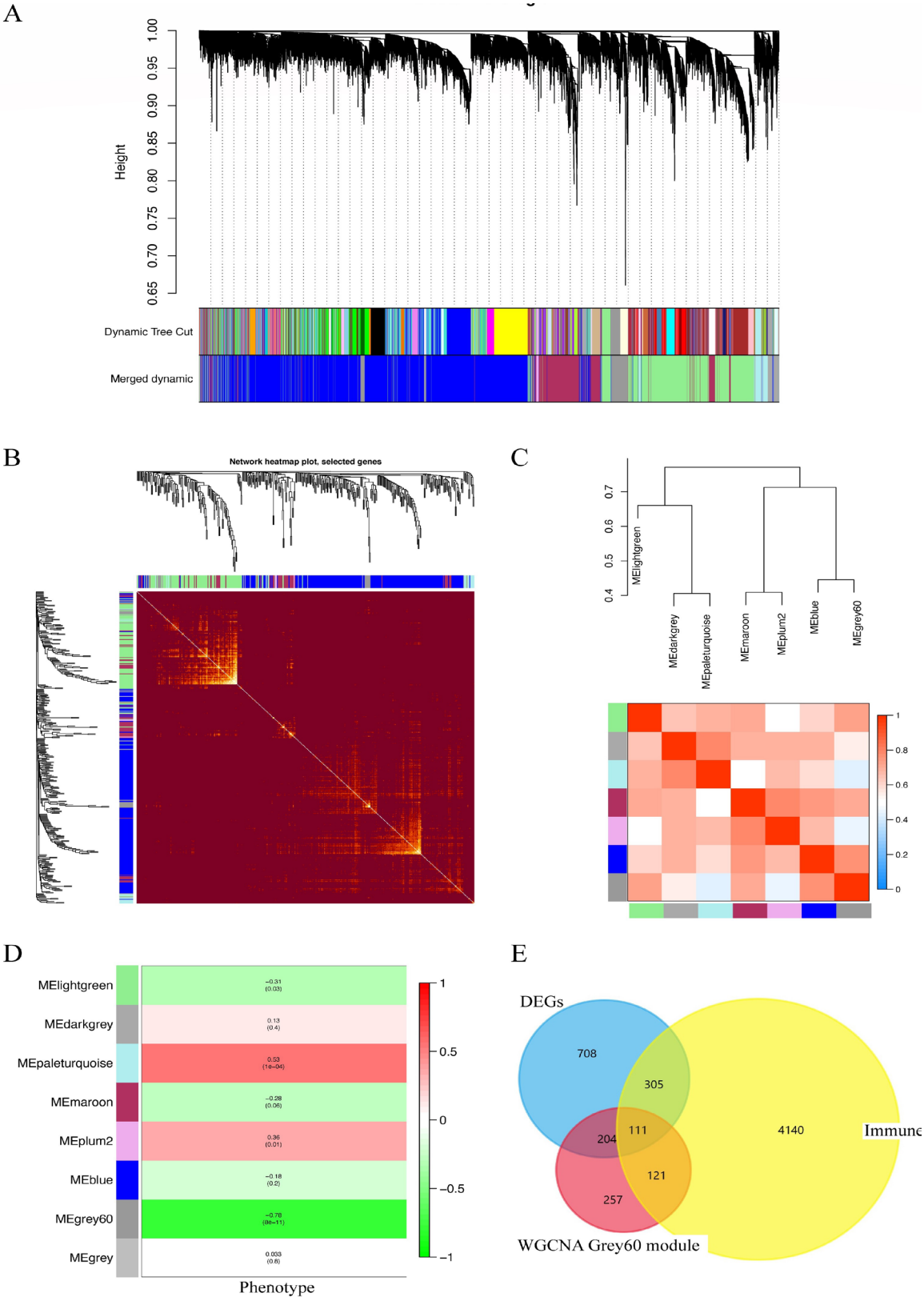

Figure 3. Construction of a weighted correlation network and identification of key modules correlated with BPH. **(A)** Dendrogram of all genes clustered based on a dissimilarity measure (1-TOM). Each color represents a module in the constructed gene co-expression network by WGCNA. **(B)** Interaction relationship analysis of co-expression genes. Different colors of horizontal axis and vertical axis represent different modules. Light color represents higher connectivity. **(C)** Clustering and correlation between module eigengenes. **(D)** Heatmap of the correlation between module eigengenes and sample phenotype. Each cell contains the correlation coefficient and P value. (E) Venn diagram of the intersection genes between the DEGs, the WGCNA Grey60 module genes and the immune gene list.

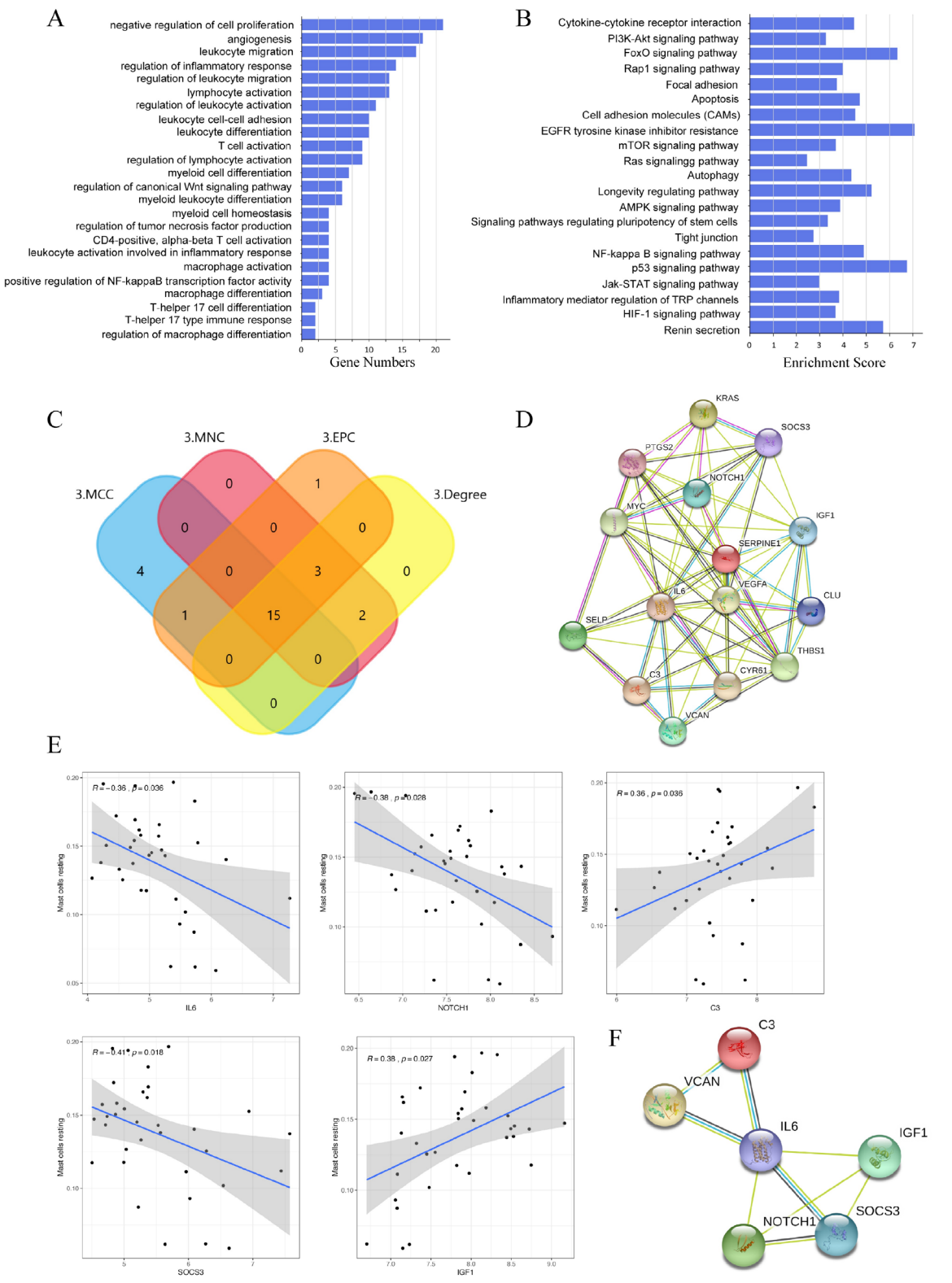

Figure 4. Identification of key regulatory genes in BPH. **(A)** Gene ontology analysis of the intersection genes. **(B)** KEGG pathway enrichment of the intersection genes. **(C)** Identification of hub genes among DEGs by the four topological algorithms, including Degree, Edge Percolated Component (EPC), Maximum Neighborhood Component (MNC), Degree method, Maximal Clique Centrality (MCC). **(D)** PPI network of key regulator of DEGs. **(E)** Relationship between mast cell subtype infiltration and hub DEGs. **(F)** PPI-network of immune-related hub DEGs: IL6, IGF1, NOTCH1, C3, VCAN and SOCS3.

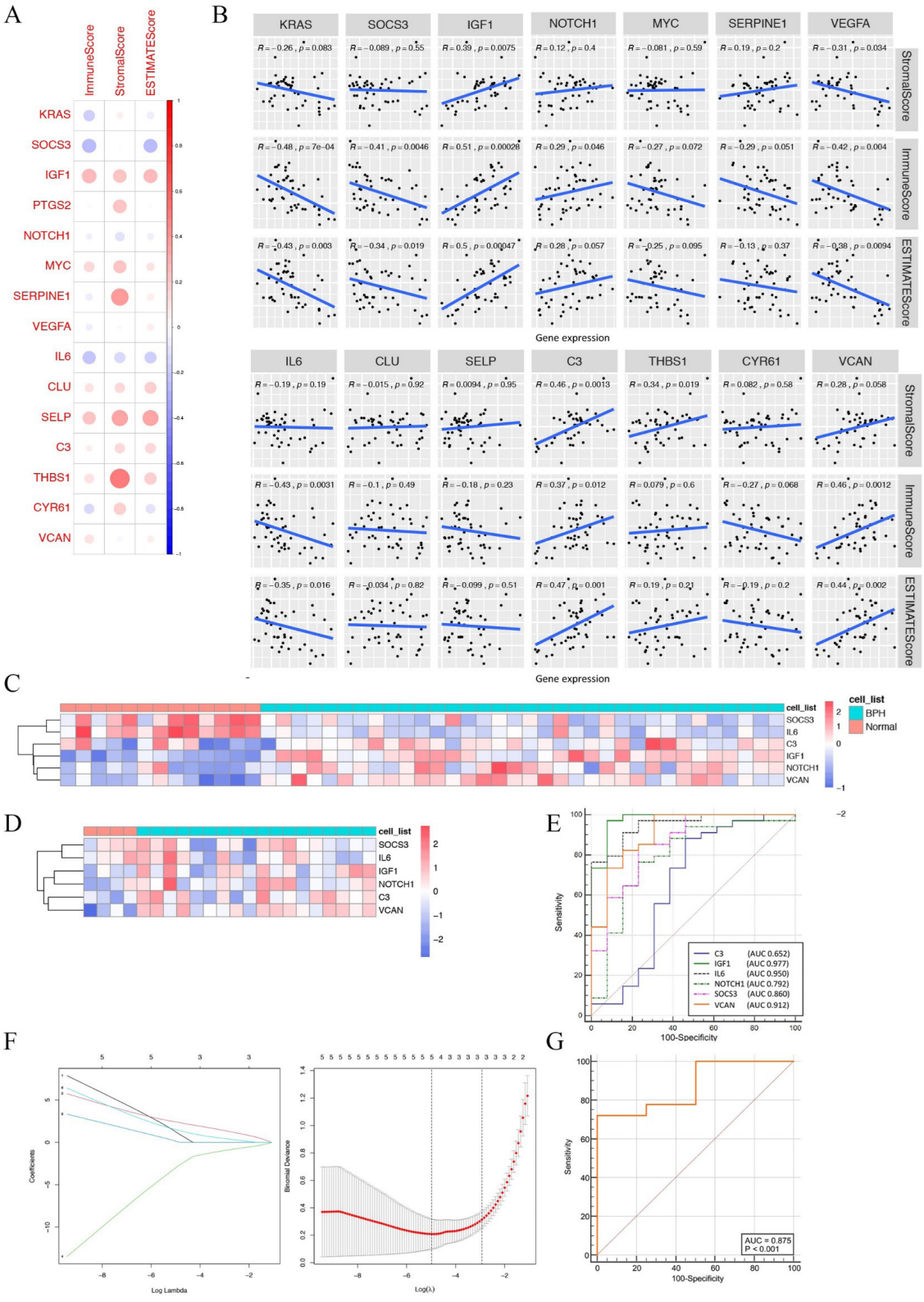

Figure 5. Establishment and validation of an immunological gene signature for BPH diagnosis. **(A)** Correlation between ESTIMATE score, immune score, stromal score and hub DEGs in BPH. **(B)** Scatter plot of relationship between ESTIMATE score, immune score, stromal score and hub DEGs. **(C, D)** Heatmap of the 6 immune-related DEGs across BPH and normal prostate tissues in the discovery cohort **(C)** and the validation cohort **(D)**. **(E)** ROC analysis of the immune-related hub DEGs for BPH diagnosis. **(F)** Construction of an immunologic gene signature for BPH diagnosis according to the expression of the immune-related hub DEGs. **(G)** ROC analysis of the immunologic gene signature for BPH diagnosis in the validation cohort.

presented higher M2 Macrophages ($P = .004$), follicular T helper cells ($P < .001$), resting mast cells ($P < .001$) infiltration. In contrast, fewer plasma cells ($P = .030$), activated CD4+ memory T cells ($P < .001$) and activated mast cells ($P = .006$) were found in BPH tissues (**Figure 2C**). The correlation between different immune cells was presented in Figure 2D. Through three-dimensional principal PCA, the proportions of these 6 types of immune cells from the tissues of BPH patients and normal controls displayed distinct group-bias clustering and individual differences (**Figure 2E**). PCA denoted that BPH tissues exhibited a unique immune cell infiltration pattern, different from normal prostate tissues.

Identification of key modules correlated with BPH by WGCNA

The input dataset for WGCNA construction consists of the common 14624 genes matrix and the phenotype of, totally, 47 samples (BPH vs. normal controls). "WGCNA" package was used in R, after quality assessment for expression matrix, R2 = 0.9 was selected to ensure a scale-free network. Then we set threshold as 0.4 to merge similar modules, and a total of 8 modules were identified (**Figure 3A**). Genes in grey module were removed in the subsequent analysis.

We calculated eigengenes of all modules and clustered them based on their correlation. Module eigengene dendrogram showed that the 7 modules were mainly divided into two clusters, and eigengene network heatmap demonstrated similar results (**Figure 3C**). Network heatmap was also performed to analyze the interaction of the 7 modules (**Figure 3B**). The results showed that grey60 module was relatively independent from other modules, indicating a high-scale independence of transcriptomic expression. Among these modules, grey60 module presented the highest relationship with the sample phenotype (**Figure 3D**). Taken together, we selected the grey60 module for subsequent analysis.

Identification of key regulatory genes in BPH.

Next, we performed differential expression analysis to identify differentially expressed genes (DEGs) between BPH and normal prostate tissues. |FoldChange|> 1 and P < .05 as the threshold, a total of 578 up-regulated and 750 down-regulated DEGs were screened out. Because BPH samples show different immunological status, we downloaded import gene list from innate database, which consist of immune-related genes.[20] After being overlapped with grey60 module from WGCNA and import gene list, the 111 genes were identified (Figure 3B). Gene ontology analysis and KEGG pathway enrichment were performed for the 111 genes to explore potential biological processes associated with BPH. Biological process of gene ontology analysis showed these genes were mainly associated with immune cell proliferation, differentiation and migration (**Figure 4A**). KEGG pathway enrichment also showed enrichment inflammation-related pathways in BPH, including cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, FoxO signaling pathway, NF-kappa B signaling pathway, mTOR signaling pathway, HIF-1 signaling pathway, and Jak-STAT signaling pathway (**Figure 4B**). PPI analysis also identified IL6, VEGFA, IGF1, SERPINE1, PTGS2, THBS1, MYC, NOTCH1, KRAS, SELP, C3, CYR61, VCAN, SOCS3, CLU as key regulatory genes of DEGs by the four topological algorithms (**Figure 4C, 4D**).

Association between key regulatory genes and immunological status in BPH

We further explored the association between hub DEGs and immunological status in BPH. Significant positive correlations were found between ESTIMATE immune Score and IGF1, NOTCH1, C3, THBS1 and VCAN (**Figure 5A, 5B**). At the meantime, negative correlations were demonstrated in KRAS, SOCS3, MYC, SERPINE1, VEGFA, CLU, SELP, CYR61 and IL6 (**Figure 5A, 5B**). However, PTGS2 showed weak association with ESTIMATE immune score (**Figure 5A**). We wonder whether infiltration of immune cells was correlated with hub DEGs. Therefore, the relationship between hub genes expression and abundance of immune cells was evaluated. The number of activated mast cells was positively associated with the expression of IL6 (R = 0.34, $P = .049$), NOTCH1 (R = 0.39, $P = .021$) and SOCS3 (R = 0.44, $P = .010$), but also negatively associated with the expression of IGF1 (R = -0.44, $P = .010$) (**Figure 4E**). On the other hand, the number of resting mast cells was positively associated with the expression of IGF1 (R = 0.38, P = .027) and C3 (R = 0.36, $P = .036$), but also negatively associated with the expression of IL6 (R = -0.36, $P = .036$), NOTCH1 (R = -0.38, $P = .028$) and SOCS3 (R = -0.41, $P = .018$) (Figure 4E). As mast cells could play a central role in allergy and regional immune response, we could infer that they are likely to play a part in the progression of BPH. In conclusion, IL6, IGF1, NOTCH1, C3, VCAN, and SOCS3 were finally identified as the immune-related hub DEGs in BPH samples (**Figure 4D**).

Establishment and validation of an immunological gene signature for BPH diagnosis

Heatmap illustrated the expression of the 6 immune-related DEGs across BPH and normal prostate tissues in the discovery cohort (**Figure 5C**) and the validation cohort (**Figure 5D**). The expression pattern of the 6 immune-related DEGs shared similar trends in both the discovery and validation cohorts. We next assessed if the 6 immune-related hub DEGs could be used for BPH diagnosis. ROC analysis showed that IGF1 (AUC = 0.977), IL6 (AUC = 0.950), VCAN (AUC = 0.912), SOCS3 (AUC = 0.860), NOTCH1 (AUC = 0.792) and C3 (AUC = 0.652) could be potential biomarkers for BPH diagnosis (**Figure 5E**). As the expression of immune-related DEGs was tightly related to immunogenic status of BPH, we further constructed an immunologic 5-gene diagnostic signature according to the expression of the immune-related hub DEGs by LASSO (Figure 5F). A final formula based on the lasso regression and expression of SOCS3, IGF1, IL6, C3 and VCAN was generated as: Signature=(1.2125546× SOCS3+2.4104945×IGF1-2.9554274×IL6+0.1217953 ×C3+1.4966001×VCAN). The signature was calculated in the validation set, according to the same formula, and validated by ROC analysis (AUC= 0.875, **Figure 5**G). This signature showed an excellent performance for BPH diagnosis according to ROC analysis. The results demonstrated the potential role of the immunological gene signature for BPH diagnosis, and may further become potential therapeutic targets.

DISCUSSION

BPH is the most common cause of bladder outlet obstruction and LUTS in aged male patients, but its molecular mechanisms have not been uncovered yet. In

this decade, genomic technologies have been applied in the discovery of BPH mechanisms. In the present study, we applied bioinformatic analysis of available transcriptomic data of BPH, and identified a distinct immune infiltration pattern of BPH, which can be helpful for BPH diagnosis and may be a new aspect for BPH treatment. We also identified the immune-related biological pathways associated with BPH development.

Although the pathogenesis of BPH has not been fully understood yet, accumulating evidences have indicated chronic inflammation as an important driver of BPH development and progression.[8,9] External stimuli could trigger dysregulated inflammation in the prostatic microenvironment. During this progress, abundant B-lymphocytes, T-lymphocytes, macrophages and mast cells may appear in the glandular, periglandular and stromal area of the prostate, and subsequent tissue damage and chronic tissue healing could result in the development of BPH.[21]

In our study, we found five subtypes of immune cells related to BPH development, including M2 Macrophages, follicular T helper cells, resting mast cells, plasma cells, activated CD4+ memory T cells and activated mast cells. In particular, the activation status of mast cells was significantly related to BPH. The infiltration of resting mast cells was enriched in BPH tissues, while the number of activating mast cells was lower in BPH tissues, compared with normal prostate tissues. Mast cells are involved in biological responses to exocrine and endocrine stimuli. The relationship between mast cells and BPH was first observed by Papadoukakis et al. in 2010.[22] They found mast cells were increased significantly in BPH model of adult Wistar rats. Further study by Ou et al. found BPH-1 prostate cells could promote migration of mast cells, while mast cell-derived interleukin-6 could conversely stimulate BPH-1 cells proliferation.[23] However, the studies of mast cells in BPH are still preliminary. Further studies of how mast cells infiltrate BPH tissues, and how they function as regulators of BPH development, are still needed.

Multiple pro-inflammatory mediators have been found involved in BPH pathogenesis, including IL-1, IL-2, IL-4, IL-8, IL-17, IFN-α and FGF-2.[24,25] Our study found up-regulated expression of C3, IGF1, NOTCH1 and VCAN, and down-regulated expression of IL6 and SOCS3 in BPH compared with normal prostate tissues. This expression pattern of BPH tissues appeared in the validation cohort as well. Since these genes mainly involved in inflammatory pathways, our findings also provided evidence for dysregulated prostatic inflammation in BPH. We also identified an immunological gene signature including these immunological genes for BPH diagnosis. This signature worked well in the external validation cohort (**Figure 5G**). Further validations are still needed for clinical application.

Androgens and androgen receptor signaling are required for the development of BPH. Steroid 5αreductase inhibitors including finasteride and dutasteride could suppress this process by blocking the conversion of testosterone to dihydrotestosterone. The shrinkage of prostate is believed to be the main effect of 5αreductase inhibitors. Recently, androgens are found to influence the development of inflammatory infiltrates in the prostate.[26] Moreover, proinflammatory cytokines might also enhance the proliferation of prostate by changing the metabolism of sex steroids and activation of AR signaling.[27] However, the complex interaction between immune cells and androgens has not been clarified yet. Our study identified a close relationship between inflammation and BPH. Since steroid 5αreductase inhibitors are one of the most common-used medication in BPH, we believe further studies are necessary to better understand the influence of AR signaling on prostatic immunity in the future.

Sequencing-based genomic analysis, including genomic, transcriptomic and epigenomic profiling, have been applied for BPH studies recently.[28-30] The studies identified several important genomic pathways important for BPH development and progression, including CXCL13, BMP5 and so on.[29] Our study integrated multiple datasets from previous studies, which provided a more comprehensive view for the pathogenesis of BPH.

However, this study still has several limitations. The limited sample size is the major limitation. Further validation in BPH cohorts with larger sample size is expected. In addition, the interplay between immune cell infiltration and therapeutic regimens of BPH was not considered in the study. Further transcriptomic studies may reveal if dihydrotestosterone blocker and □-adrenoreceptor antagonist therapy would change the BPH microenvironment, and if the microenvironment has impact on the effect of medical treatment in BPH. Moreover, this study is only a preliminary exploration of the mechanism between immune microenvironment and BPH progression. Interventional and functional studies are needed to fully understand the underlying mechanisms.

CONCLUSIONS

This study applied a comprehensive transcriptomic approach to identify immunologic changes in BPH. BPH samples exhibited a characteristic immune infiltration pattern, with more resting mast cell and less activating mast cell infiltration. We also identified a novel immunological gene signature, which can be helpful for BPH diagnosis. These findings need to be confirmed in larger prospective datasets.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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