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Original Article

Screening of key genes associated with m6A methylation in diabetic nephropathy patients by CIBERSORT and weighted gene coexpression network analysis

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Abstract: Diabetic nephropathy (DN) is a common complication of diabetes. Due to its complex pathogenesis, there is no effective treatment. M6A is a newly discovered epigenetic mechanism that may be involved in the development of diabetic nephropathy. In this study, we analyzed differentially expressed genes (DEG) in the GEO database (GSE96804) and paid attention to genes with m6A methylation. 623 DEGs in glomerular tissue were identified by comparing diabetic nephropathy with normal. Correlation analysis with 21 genes involved in m6A modification showed that 492 genes were associated with m6A methylation. According to the CIBERSORT algorithm, the infiltration of M1 macrophages in DN patients was significantly higher than that in normal samples. Weighted gene coexpression network analysis (WGCNA) was used to screen for the modules most correlated with the clinical features of M1 macrophages. The genes in the selected modules and 492 m6A-related DEGs were intersected by a Venn diagram, and 43 key genes were obtained. GO and KEGG analyses showed that these genes were mainly related to the positive regulation of protein aggregation and the transforming growth factor β receptor signaling pathway. According to a literature review, among the top 10 genes, HSPA1A, HSPA1B, CHI3L1, TYRO3 and PTH1R are markers in diabetic nephropathy, and their abnormal expression is associated with renal hypertrophy, proteinuria and glomerulosclerosis. These findings may provide evidence for the diagnosis and treatment of diabetic nephropathy.

Keywords: Diabetes, diabetic nephropathy, m6A, weighted gene co-expression network, differential analysis, CIBERSORT, enrichment analysis

Introduction

Diabetes is a serious disease that endangers human health worldwide, and its incidence is increasing gradually [1]. According to the International Diabetes Federation, the number of adults worldwide with diabetes was estimated at 537 million in 2021 and is expected to grow to 643 million in 2030 [2]. Diabetic nephropathy is one of the common complications of diabetes and a common cause of end-stage renal disease [3]. In recent years, the number of diabetic nephropathy patients in China has increased. At present, blood pressure lowering and hypoglycemic and lipid regulation are the main means of treating diabetic nephropathy domestically and overseas [4]. Although they can delay the disease, there is no specific therapy for reversion or eradication. Therefore, it is urgent to further explore its pathogenesis to find effective biomarkers for its early diagnosis and treatment.

Epigenetics regulates gene expression independently of genome sequence and plays an important role in various diseases and tumors. In general, epigenetic regulation refers to the diversified and reversible chemical modification of DNA and histones [5]. In addition to DNA and histones, there are also different types of post-transcriptional modifications of RNA (mRNA, lncRNA, snRNA, etc.) [6]. M6A methylation, one

of the most common epigenetic mechanism, refers to the addition of a methyl group to the sixth nitrogen atom of adenine to form 6-methyladenine [7]. Approximately 0.1-0.4% of adenosine in total RNA is modified by m6A methylation. As the most abundant post-transcriptional modification at the RNA level, m6A methylation is a dynamic and reversible modification process mediated by m6A WER ("Writers", "Erasers" and "Readers") proteins [8]. Methyltransferases (writers), which are mainly responsible for adding methyl to specific sites of mRNA of specific target genes, and are composed of catalytic subunit methyltransferase like 3 (METTL3), its methyltransferase like 14 (METTL14) and WTAP (Wilms' tumor 1-associated protein) [9]. Demethylases (erasers) include proteins such as ALKBH5 and FTO. Their function is to recognize m6A methylation and to remove it [10]. In addition, YTHDF and YTHDC family members are generally regarded as m6A methylated readers [11], which can specifically recognize and bind m6A methylation sites and induce their corresponding functions. There is increasing evidence that m6A modification plays an important biological function in mammals. The m6A WER protein also plays a very important role in diabetic nephropathy. METTL14 is significantly overexpressed in the glomerular endothelial cells of patients with diabetic nephropathy and plays an important role in diabetic nephropathy by altering the m6A methylation of α-klotho [12]. Furthermore, METTL3 was increased in renal biopsy podocytes in diabetic nephropathy patients and was associated with renal injury. Knockout of METTL3 significantly reduced high glucose-stimulated podocyte inflammation and apoptosis [13].

It has been reported that regulatory T cells of type 1 diabetes are significant defects [14]. By culturing them in vitro, they can significantly improve cell function and maintain the diversity of T cell receptors, which is beneficial for reducing the damage to β cells through autoimmunity. However, the core pathogenesis of type 2 diabetes is insulin resistance, which is more complex than type 1 diabetes [15]. Chronic low-grade inflammation is closely related to insulin resistance and is also involved in diseases such as atherosclerosis associated with insulin resistance, suggesting that chronic low-grade inflammation related to immune factors is also involved in the occurrence and development of type 2 diabetes and its complications. Whether the immune system involved in chronic low-grade inflammation has m6A modification, which promotes the occurrence and development of diabetic nephropathy, has not been studied.

CIBERSORT is a bioinformatics algorithm developed by Newman et al. The cell types and immune cell composition of tested samples can be identified by estimating relative subsets of RNA transcripts and complex tissue-standardized gene expression data, and then the proportion and number of immune cells in the samples can be analyzed. The algorithm has been fully applied to the immunotherapy of various diseases and tumors.

Weighted gene co-expression network analysis (WGCNA) is a comprehensive analysis technology based on biological networks. It can identify a class of co-expressed genes (or proteins) and use algorithms to associate the clustering module with the phenotype to explore the core genes (or proteins) in the module. The protein interaction network is composed of the interactions between various proteins and participates in biological processes such as signal transmission, gene expression regulation, and material metabolism [16]. It can be used for multigene analysis of complex mechanisms and large data sets, as well as to reveal the associations between genes in different samples.

In this paper, the glomerular transcriptome data of 41 patients with type 2 diabetic nephropathy and 20 normal healthy samples were obtained from the GEO database (GSE96804). The correlation between differentially expressed genes and m6A was analyzed. Combined with the CIBERSORT algorithm and weighted gene co-expression network analysis, we screened differentially expressed genes related to m6A methylation modification in macrophages of DN patients. GO and KEGG analyses were performed to provide a theoretical basis for the diagnosis and treatment of DN patients.

Materials and methods

Data collection and processing

By searching the GEO database (https://www.ncbi.nlm.nih.gov/geo/) for keywords such as

homo, diabetic nephropathy, and glomerulus, there were 61 samples in this dataset (GSE96804), including 41 diabetic nephropathy samples and 20 normal controls. All disease samples were type 2 diabetes mellitus, and all samples were from glomeruli. According to the annotation information of the GPL17586 platform (Affymetrix human transcript array 2.0), the probes were converted into the corresponding gene symbol.

DEG analysis

To identify differentially expressed genes (DEGs) in the glomerular samples of healthy people and diabetic nephropathy patients, two groups of normalized DEGs were identified using R language Limma software. The Mann-Whitney test was used to determine the differential expression levels of genes between diabetic nephropathy patients and corresponding control samples. |log2FC|>1 and P<0.05 were considered statistically significant.

M6A-related genes identification

To identify whether differentially expressed genes between diabetic nephropathy patients and healthy normal samples were associated with m6A-related genes, m6A-related DEG genes were identified by Pearson correlation analysis (|Pearson R|>0.5, p<0.05).

Immune infiltration analysis

The CIBERSORT algorithm was used for analysis, and the deconvolution method was used to process marker gene expression values to estimate the proportion of various types of immune cells in normal and diabetic nephropathy glomerular tissues. These cells include M1 macrophages, M2 macrophages, plasma cells, static memory CD4 T cells, γδT cells and mast cells, which are 22 kinds of immune cells. A boxplot of the proportion of each immune cell was drawn for all samples. Green represents the proportion of immune cells in diabetic nephropathy samples, and red represents the proportion of immune cells in normal samples. R package Nortest is used to test normality of data through AD test. Having tested that the data follow the normal distribution, the t-test method is used to check whether the results are statistically significant and marked at the top of the figure.

Construction of weighted gene co-expression network analysis

To explore the modules and genes related to the clinical characteristics of immune cells in the samples of diabetic nephropathy patients, the top 50% of genes with large gene changes were screened to cluster the samples, and the outlier samples (GSM2544295 and GSM2544309) were eliminated. The "pickSoftThreshold" function in the WGCNA package was used to calculate the correlation coefficient of the β value and the mean value of gene connectivity as the soft threshold for subsequent network construction. Then, the topological overlap matrix (TOM) is constructed by using the blockwise modules function. The dynamic tree cutting algorithm is used for clustering. The genes with similar expression patterns were grouped into the same co-expression module, and each module was assigned a different color to distinguish them. Among them, the minimum number of genes in the module is 30, the threshold of similar module merging is 0.2, and default parameters are used for the rest. Finally, gene significance (GS) and module membership (MM) were calculated, associating modules with clinical traits. The module with the highest correlation with macrophages was selected, and the genes in the module were analyzed in the next step. Genes in the co-expression module have high connectivity, and genes in the same module may have similar biological functions.

Venn diagram and enrichment analysis

Venn plots (https://bioinfogp.cnb.csic.es/tools/venny/) were used to analyze the intersection of DEGs and m6A modification-related genes in immune cells. GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses were performed, and the significance threshold was P≤0.05. GO analysis was used to annotate the functions of genes and their products in biological processes (BP), molecular functions (MF) and cellular components (CC); the KEGG database contains information on genes, proteins, chemical components and their interactions, reactions and relationship networks and can be used to annotate and analyze gene functions and metabolic pathways.

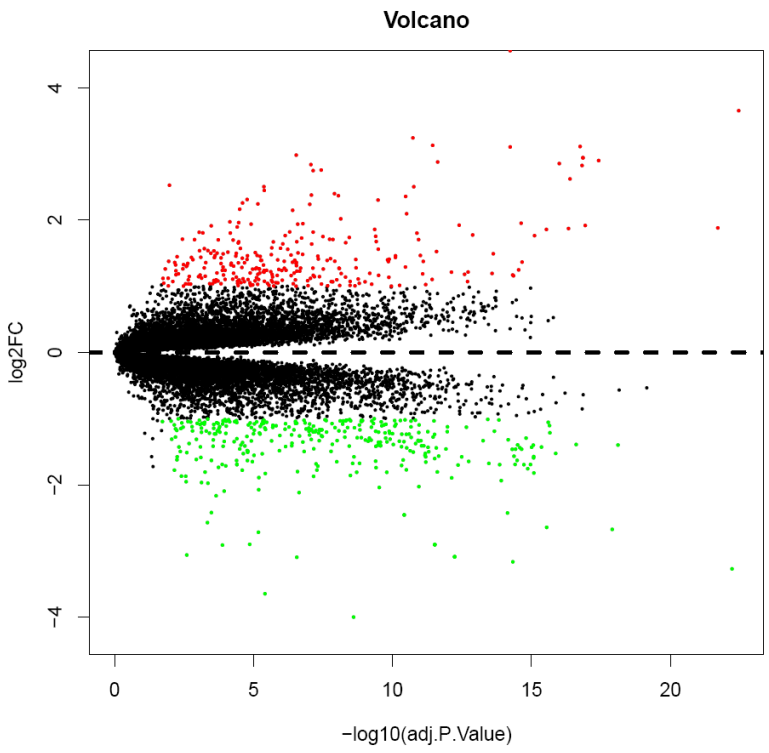

Figure 1. Screening of differentially expressed genes in diabetic nephropathy patients and healthy controls in the GSE96804 dataset; DEG analysis Volcano map of differentially expressed genes (red dots represent up-regulated genes, green dots represent down-regulated genes).

Results

Screening for differentially expressed genes

First, the R package Limma was used to screen the differentially expressed genes, and the screening criteria |log2FC|>1, adj.P. Val<0.05 (correction method is FDR) as the threshold [17]. A total of 623 differentially expressed genes were screened from the GSE96804 data set between T2D diabetic patients and healthy normal samples. There were 283 up-regulated genes and 340 down-regulated genes (**Figure 1**). DEG results were used to draw a volcano map, with red representing up-regulated genes and green representing down-regulated genes.

Screening of m6A-related genes

To investigate whether differentially expressed genes in diabetic patients are associated with m6A methylation. We used Pearson correlation analysis to screen RNA related to 21 genes involved in m6A modification (YTHDF2, RBM15, IGFBP2, LRPPRC, RBM15B, METTL14, YTHDC2, IGFBP1, HNRNPA2B1, IGFBP3, YTHDF3, FMR1, RBMX, ZC3H13, HNRNPC, METTL3, FTO, ALKBH5, METTL16, YTHDF1, YTHDC1). The screening conditions were set as a correlation coefficient >0.5 and P<0.05, and a total of 492 m6A-related genes were identified (**Figure 2**). As shown in the network diagram, the red dot represents the genes related to m6A, and the blue dot represents the differentially expressed genes related to m6A. In summary, 492 genes in this dataset are abnormally expressed in diabetic nephropathy patients and may play a role through m6A methylation modification.

Assessment of infiltrating immune cells in diabetic nephropathy

At the same time, we analyzed the infiltration of immune cells in the above samples of normal and diabetic nephropathy. We selected 20 normal samples and 41 diabetic nephropathy samples. The CIBERSORT algorithm was used to analyze the data, and a boxplot of the proportion of 22 immune cells was drawn in R language, where red represents normal samples and green represents diabetic nephropathy samples (**Figure 3**). As shown in the figure, the proportion of M1 macrophages in diabetic nephropathy tissue samples was significantly higher than that in the control group. This suggests that macrophages and other immune factors may play a very important role in the occurrence and development of DN.

WGCNA module construction and screening

WGCNA technology is a high-throughput data mining algorithm for analyzing gene expression data [18]. Compared with the one-dimensional molecular biology research method, the gene modules constructed by this method cover almost all human genes, showing a more accurate biological system. To evaluate the modules with the highest correlation with immune cells

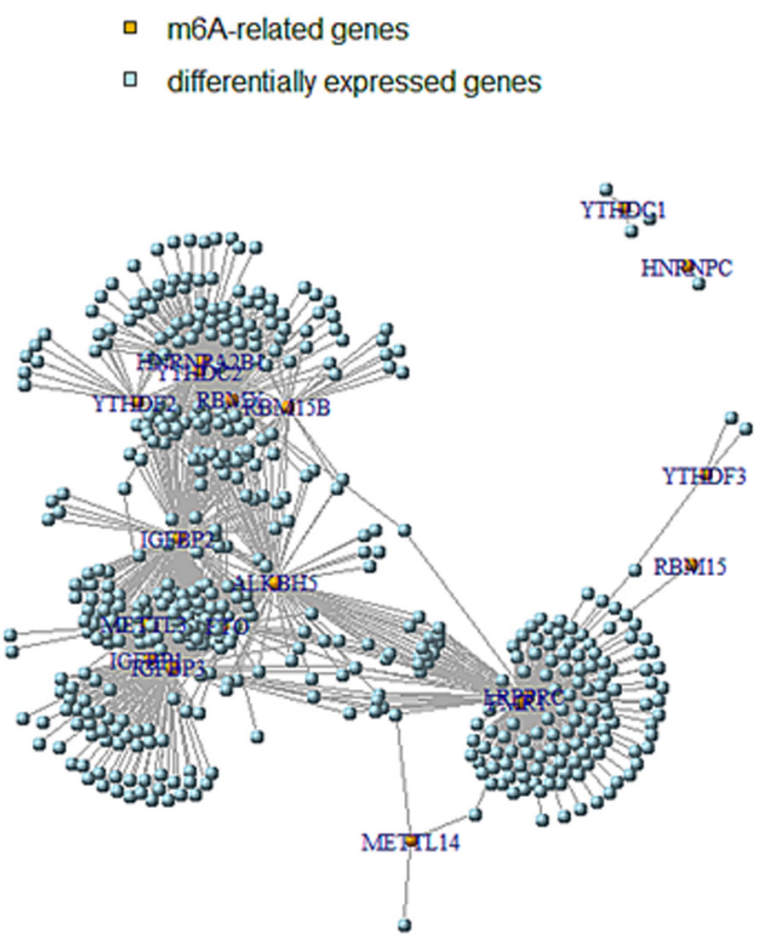

Figure 2. Screening of DEGs associated with m6A. The orange dots represent the m6A-related genes, the green dots represent the DEGs, and the lines represent the correlations between the dots.

in diabetic nephropathy patients, the WGCNA package in R language was used to construct the gene co-expression network, identify the modules, and analyze the relationship between sample characteristics and modules. After setting the height to 94, two outlier samples were removed, and the remaining samples were retained for further analysis (**Figure 4A**). When the scale-free topological fitting index R2 was adjusted to 0.9, the appropriate β value was 3 (**Figure 4B**). The dynamic clipping tree algorithm was used to segment modules and construct a network graph (**Figure 4C**). Cluster analysis is carried out on modules, and the modules with similar distances are merged into new modules. The abscissa represents various types of immune cells, and the color blocks on the ordinate represent different modules. In the heatmap of the middle part, the darker the color is, the higher the correlation. Red represents a positive correlation, while blue represents a negative correlation (**Figure 5A**). Due to the significant difference in the proportions of M1 macrophages between diabetic nephropathy patients and normal samples, we will focus on the red module of M1 macrophages genes in subsequent studies (**Figure 5B**).

Search for key genes associated with both m6A and macrophages

To identify differentially expressed genes associated with macrophages and modified by m6A, we used the online website https://bioinfogp.cnb.csic.es/tools/venny to draw a Venn diagram (**Figure 6A**). The 492 DEGs associated with m6A were intersections with 1212 genes of the most related modules of macrophage M1 Thus, 43 (M1) differentially expressed genes associated with m6A methylation were obtained. Metascape was used to conduct enrichment analysis for m6A-related differential genes intersecting M1 (**Figure 6B**). Macrophage M1 is generally believed to cause kidney damage and renal fibrosis in patients with diabetic nephropathy and to secrete excessive inflammatory factors to damage kidney tissue [19], so we focused on M1 macrophages. The results of enrichment analysis showed that these genes were mainly enriched in the positive regulation of protein aggregation, the regulation of the transforming growth factor β receptor signaling pathway, the regulation of systemic processes, and lung development. Analysis of their pathways revealed that HSPA1A, HSPA1B, NPHS1, TPPP3, TYRO3, CHI3L1, DACH1, PTH1R, LOX, PRKAR2B and other genes were abnormally expressed by m6A methylation in diabetic nephropathy patients and were significantly correlated with macrophage infiltration in the glomerular tiss

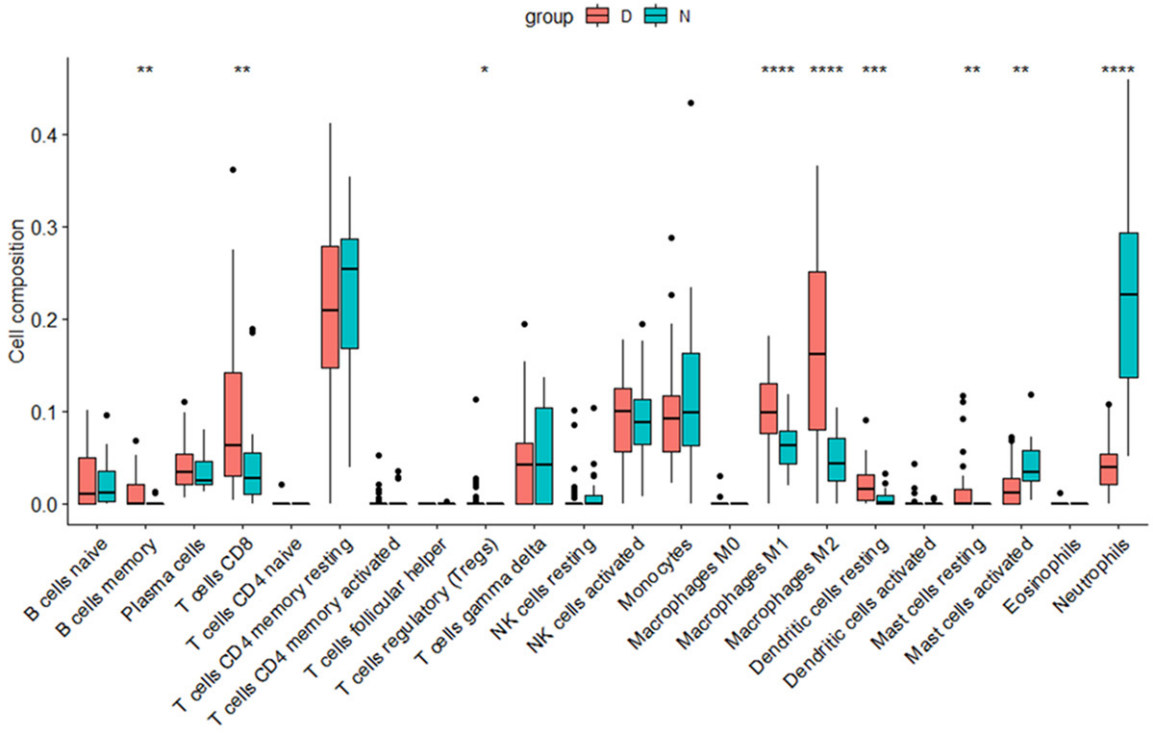

Figure 3. Boxplot of the proportion of immune cells in diabetic nephropathy patients and normal samples. The abscissa is the type of immune cells, and the ordinate is the proportion of immune cells. Green represents the proportion of immune cells in diabetic nephropathy samples, red represents the proportion of immune cells in normal samples, 0.01<P≤0.05 marked as "*", 0.001<P≤0.01 marked as "**", 0.0001<P≤0.001 marked as "***", and P≤0.0001 marked as "****".

ue of patients, which is worth further discussion.

Discussion

As a common chronic complication of diabetes, diabetic nephropathy can cause glomerular hypertrophy, basement membrane thickening, glomerulosclerosis and renal interstitial fibrosis, eventually leading to renal failure [20] and seriously endangering human life and health safety. Due to the limited understanding of its pathogenesis and treatment, it is necessary to develop new biomarkers and potential targets at the molecular level to prevent and treat diabetic nephropathy. In this study, we used the GSE96804 dataset in the GEO database and the R language CIBERSORT algorithm to analyze the infiltration of immune cells. We found that the proportions of M1 macrophage and other immune cells in glomerular samples from diabetic nephropathy patients were significantly higher than those in normal samples. The above immune cells were analyzed by the WGCNA module and intersected with m6A-related differentially expressed genes. A total of 43 genes that were significantly related to the M1 phenotype of macrophages and involved in m6A modification were screened.

It has been reported that macrophage infiltration is one of the prominent features of diabetic nephropathy and is significantly increased in the glomerular tissue of most diabetic nephropathy patients. As an important part of the mononuclear phagocyte system, macrophages play a very important role in body, such as development, tissue homeostasis and repair. Generally, macrophages have two phenotypes, namely, M1 macrophages and M2 macrophages, which have different functions. M1 macrophages are typically activated cells that produce inflammatory cytokines [21], which can secrete excessive inflammatory cytokines (such as IL-1β and IL-23), chemokines and reactive oxygen species to promote inflammatory responses, thus damaging the kidneys of patients with diabetic nephropathy. However, M2

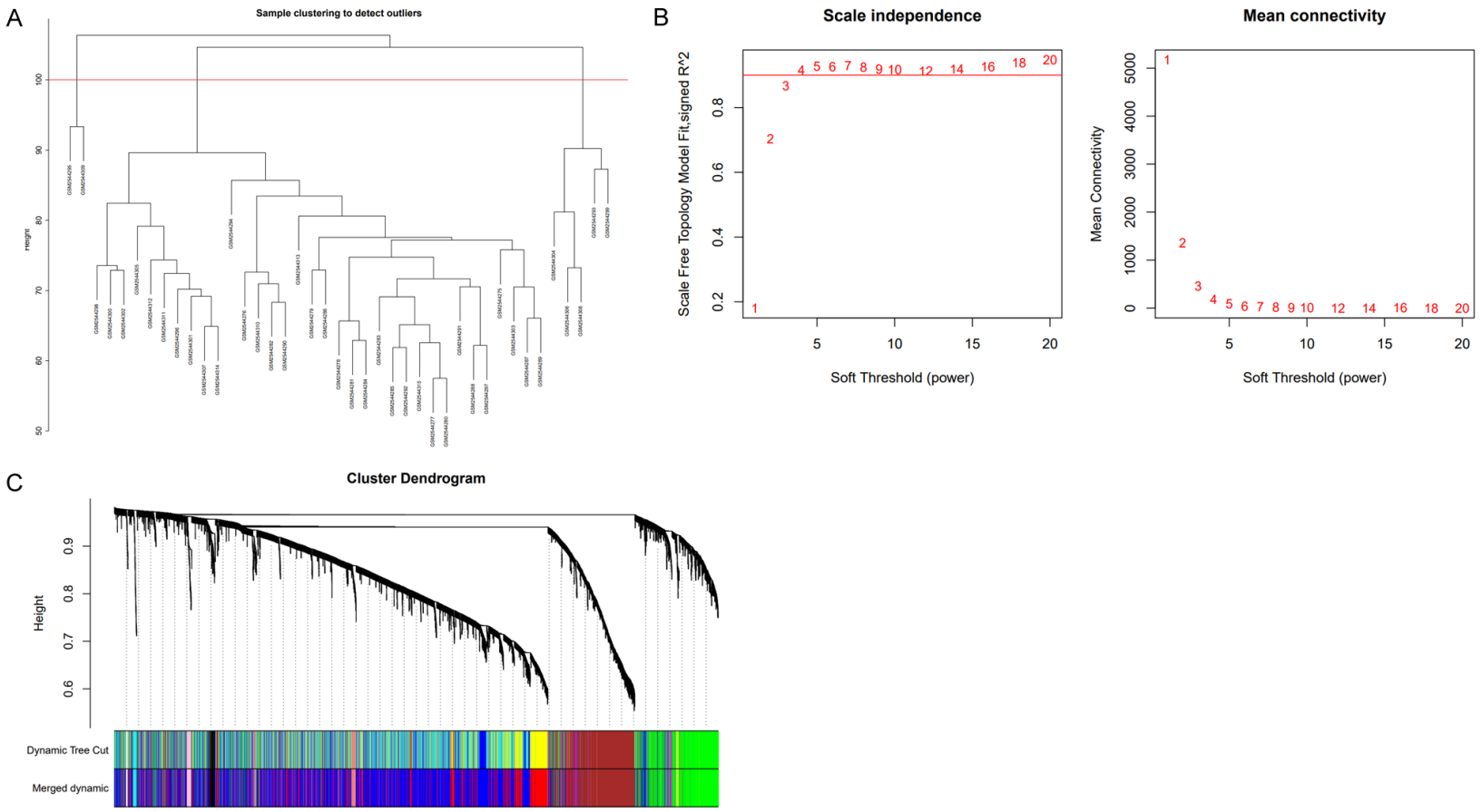

Figure 4. Construction of the co-expression network. A: Sample clustering diagram (delete 2 outlier samples by setting the height to 94); B: Determination of the optimal soft threshold (in the process of module selection, the adjacency matrix is converted into a topology matrix, and the optimal soft threshold β=3 is determined); C: Cluster tree of coexpressed gene modules (similar genes are grouped into the same module through dynamic splicing and cluster analysis).

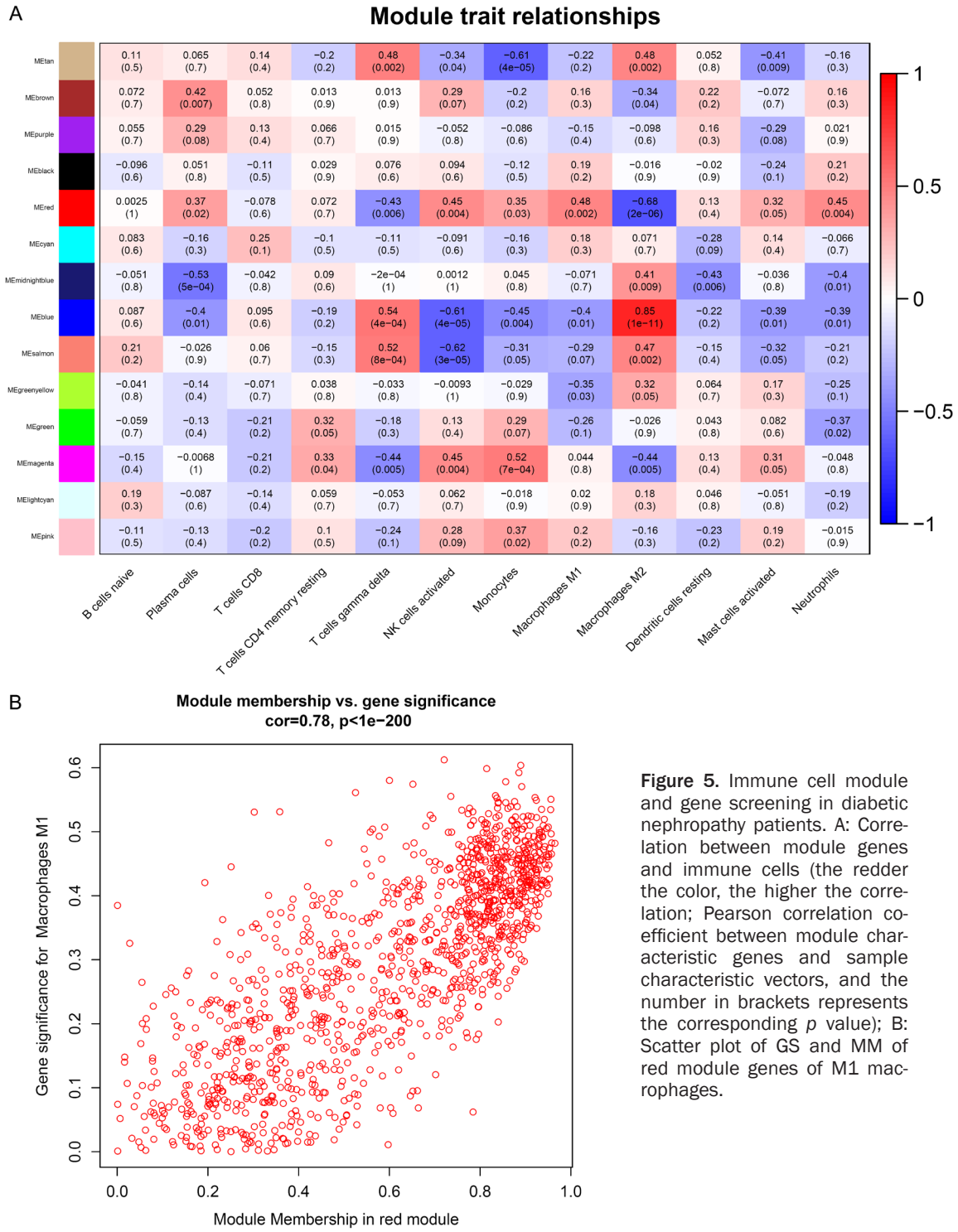

Figure 5. Immune cell module and gene screening in diabetic nephropathy patients. A: Correlation between module genes and immune cells (the redder the color, the higher the correlation; Pearson correlation coefficient between module characteristic genes and sample characteristic vectors, and the number in brackets represents the corresponding *p* value); B: Scatter plot of GS and MM of red module genes of M1 macrophages.

macrophages usually act as anti-inflammatory cells [22] and are involved in immunosuppression, tissue remodeling, and tumor progression. Therefore, we focused on the significantly increased M1 macrophages in glomerular samples from patients with diabetic nephropathy.

The 492 m6A-related DEGs were intersected with the 1212 key genes in the red module by a Venn diagram, and 43 candidate genes were obtained. After GO enrichment analysis, we found that the above candidate genes were significantly enriched in the positive regulation

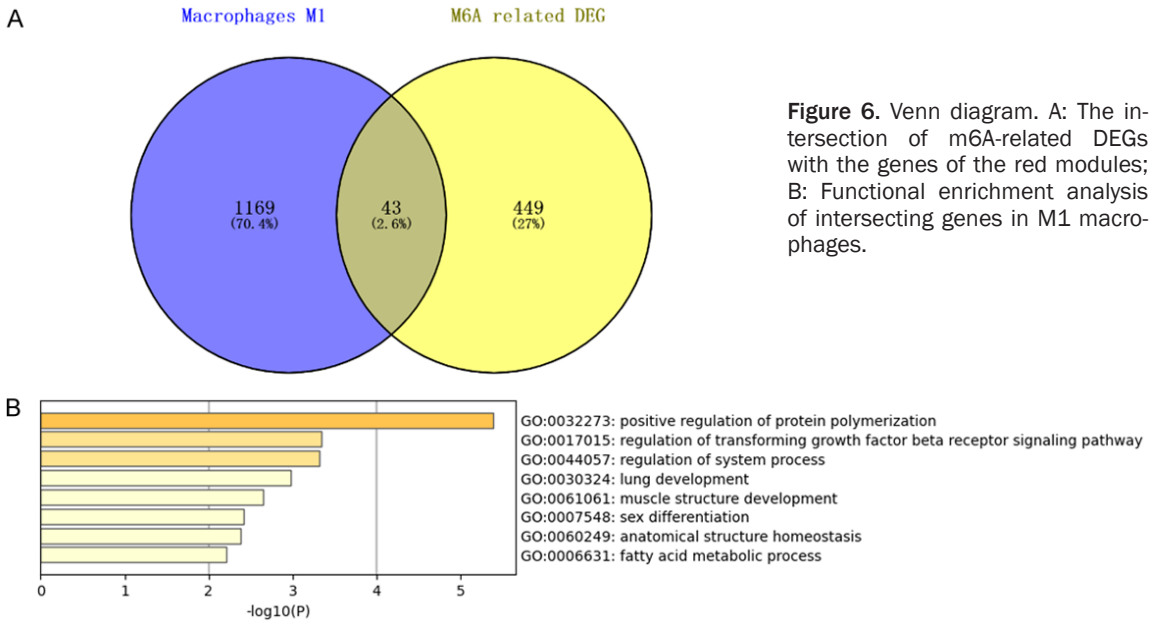

Figure 6. Venn diagram. A: The intersection of m6A-related DEGs with the genes of the red modules; B: Functional enrichment analysis of intersecting genes in M1 macrophages.

of protein aggregation, transforming growth factor β receptor signaling pathway and other pathways, suggesting that these candidate genes may influence the development of diabetic nephropathy through regulatory protein regulation and growth factor β. Next, we selected the top 10 genes with the most differential expression in the positive regulatory pathway of protein aggregation, namely, HSPA1A, HSPA1B, NPHS1, TPPP3, TYRO3, CHI3L1, DACH1, PTH1R, LOX and PRKAR2B, for further analysis.

We defined the above genes as key genes involved in m6A methylation modification in M1 macrophages of DN patients. However, it is not clear whether these genes are involved in kidney damage in DN patients through m6A methylation modification and whether they can be used to guide the early diagnosis and treatment of diabetic nephropathy. A literature review showed that among the 10 genes mentioned above, PTH1R and HSPA1A could affect osteoblast differentiation through m6A methylation modification. HSPA1A interacts with the m6A demethylase FTO to enhance the stability of mRNA proteins that protect cells from genotoxic damage. In addition, METTL3-mediated m6A methyltransferase targets PTH1R and reduces protein translation, affecting osteoblast differentiation. These results indicate that the abovementioned genes may regulate diabetic nephropathy through m6A modification, which should be discussed in the future.

Next, we analyzed the expression changes of these genes and found that they were significantly upregulated in DN patients. Moreover, it has been reported that HSPA1A and HSPA1B have gene polymorphisms in diabetic nephropathy patients and are significantly related to susceptibility to diabetic nephropathy [23]. In addition, NPHS1 encodes epinephrine, and its abnormal expression and mutation are associated with increased proteinuria in diabetic patients [24]. Other studies have reported that CHI3L1 is significantly upregulated in atherosclerosis, coronary artery disease, acute ischemic stroke, kidney disease, diabetic retinopathy and other diseases and is considered a noninvasive prognostic biomarker of inflammation and may also play a similar role in diabetic nephropathy [25]. The above conclusions indicate that the key genes related to m6A and involved in macrophage M1 infiltration screened by bioinformatics methods are significantly correlated with the occurrence and development of diabetic nephropathy. These genes may be associated with m6A methylation and infiltration of immune cells, especially M1 macrophages, and thus provide new targets for the diagnosis and treatment of DN patients.

However, this study also has some limitations. Due to the small number of samples in the GSE96804 dataset and the lack of clinical information, it cannot cover all diabetic nephropathy patients. In the future, we will further explore the data related to diabetic nephropa-

thy and its survival information and combine the conclusions of this paper to carry out further clinical verification of the screened related genes. Second, among the genes screened in this paper, only two genes, PTH1R and HSPA1A, have been clearly reported to have mRNA expression regulated by m6A methylation. In the osteoblast, the increased m6A methylation level of PTH1R affects its mRNA stability and leads to reduced translation level, thus damaging the PTH-PTH1R signaling pathway and ultimately leading to osteoblast disorder [26]. In contrast, in preeclampsia, HSPA1A is significantly upregulated by m6A methylation and is involved in the progression of preeclampsia [27]. Therefore, in a follow-up study, we will experimentally confirm whether other key genes can affect expression by changing m6A methylation modification. To determine whether it can be used as a new therapeutic target and provide a theoretical basis for the clinical diagnosis and treatment of diabetic nephropathy.

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Disclosure of conflict of interest

None.

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