Recognition of differently expressed genes and DNA methylation markers in patients with Lupus nephritis

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ABSTRACT

Background and Objectives: Systemic lupus erythematosus (SLE) is distinguished by dysregulated immune system activity, resulting in a spectrum of clinical manifestations, with lupus nephritis being particularly prominent. This study endeavors to discern novel targets as potential therapeutic markers for this condition. **Methods:** Weighted correlation network analysis (WGCNA) was used to construct the network and select the key hub genes in the co-expression module based on the gene expression dataset GSE81622. Subsequently, functional enrichment and pathway analysis were performed for SLE and lupus nephritis. In addition, also identify genes and differences in SLE with lupus nephritis and methylation site. Finally, qRT-PCR and western blot were used to verify the up-regulated expression levels of the selected key genes. **Results:** Within the co-expression modules constructed by WGCNA, the MElightcyan module exhibited the strongest positive correlation with lupus nephritis (0.4, P = 0.003), while showing a weaker correlation with the control group SLE (0.058) and a negative correlation with the control group (-0.41, P = 0.002). Additionally, the MEgreenyellow module displayed the highest positive correlation with SLE (0.25), but its P value was 0.06, which did not reach statistical significance(P > 0.05). Furthermore, it had a negative correlation with the control group was (-0.38, P = 0.004). The module associated with lupus nephritis was characterized by processes such as neutrophil activation (neutrophil_activation), neutrophil degranulation (neutrophil_degranulation), neutrophil activation involved in immune response (neutrophil_activation_involved_in_immune_response), neutrophils mediated immune (neutrophil_mediated_immunity) and white blood cells degranulation (leukocyte_degranulation) and so on the adjustment of the process. Secondly, in the analysis of SLE samples, the identification of differentially expressed genes revealed 125 genes, with 49 being up-regulated and 76 down-regulated. In the case of lupus nephritis samples, 156 differentially expressed genes were discerned, include in 70 up-regulated and 86 down-regulated genes. When examining differential methylation sites, we observed 12432 such sites in the SLE sample analysis, encompassing 2260 hypermethylation sites and 10172 hypomethylation sites. In the lupus nephritis samples analysis, 9613 differential methylation sites were identified, comprising 4542 hypermethylation sites and 5071 hypomethylation sites. Substantiating our findings, experimental validation of the up-regulated genes in lupus nephritis confirmed increased levels of gene expression and protein expression for CEACAM1 and SLC2A5. **Conclusions:** We have identified several genes, notably CEACAM1 and SLC2A5, as potential markers for lupus nephritis. Their elevated expression levels and reduced DNA methylation in lupus nephritis contribute to a more comprehensive understanding of the aberrant epigenetic regulation of expression in this condition. These findings hold significant implications for the diagnosis and therapeutic strategies of lupus nephritis.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a prevalent autoimmune disease worldwide, impacting various organs in adults, notably the kidney and cardiovascular system.[1] It exhibits a higher prevalence among young women, with an increasing occurrence of early, mild and atypical cases.[2,3] SLE is characterized by a propensity for relapse and carries a substantial burden of disability and mortality.[4,5] Renal involvement, referred to as lupus nephritis (LN), is widely recognized as the most severe complication, as it is associated with progression to end-stage renal disease (ESRD) and increased mortality rates.[6,7] Lupus nephritis is diagnosed in approximately 38.5% of individuals newly diagnosed with SLE,[8] with many cases occurring within the first five years of the disease.[9] As the disease advances, approximately 60% of SLE patients develop complication.[10,11] In a study conducted on hospitalized patients SLE patients, lupus nephritis was the most commonly affected organ over a four-years affected organs.[12] However, effective treatment for SLE patients remain elusive, imposing a substantial burden on individuals and society. Therefore, a comprehensive exploration of the pathogenesis of SLE and the identification of key targets and specific markers hold immense significance for the treatment and early screening of SLE, particularly in cases involving nephritis (SLE-LN).

In recent studies, high-throughput gene chip platforms have been extensively utilized to delve into and identify promising genomic-level biomarkers for disease diagnosis and prognosis.[13] A plethora of studies have underscored the intimate connection between the pathological and physiological process of SLE and gene mutation as well as abnormal expression. Noteworthy genes in this context include TNFSF4, NCF1-339 and CXorf21.[14-16] Previous investigations have spotlighted the significance of IFI44L promoter methylation as a blood-based biomarker for systemic lupus erythematosus.[17] In an animal study, researchers established a correlation between IFIT1 expression in podocytes of MRL/lpr mice and the resultant renal pathological changes it instigates.[18] Moreover, the abnormal elevation of IFIT3 has been linked to the overactive cyclic GMP-AMP synthase/interferon gene signaling in monocytes of individuals with systemic lupus erythematosus.[19]

Alterations in the expression of immune markers, including Tyrobp, C1qb, LapTM5, CTSS and PTPRC, have been found to be closely associated with the disease pattern of lupus nephritis.[20] Furthermore, it has been demonstrated that cGAS activation in TREX1 mutant mouse models precipitates lupus-like autoimmune disease.[21] Additionally, IRF5 risk variants have been connected to heightened IRF5 expression and increased IFN production in blood cells of individuals with SLE.[22] Consequently, it is of paramount importance to explore precise molecular targets and biomarkers implicated in the onset and progression of SLE, with the aim of advancing the diagnosis and treatment of this condition.

A multitude of epigenetic phenomena, encompassing non-coding RNA regulation, chromatin remodeling, histone modifications, and DNA methylation, have been documented as significant contributors to the pathophysiological mechanisms of autoimmune diseases.[23] Among these, DNA methylation stands out as a well-established and extensively studied epigenetic factor. It has been implicated in the regulation of mRNA and miRNA expression.[24] Notably, a growing body of research has elucidated the association between aberrant DNA methylation within gene promoter regions is associated with the pathogenesis of SLE.[25-27] Recent research has indicated that DNA methylation of IFI44L may serve as a potential blood biomarker for childhood-onset systemic lupus erythematosus.[28] Additionally, a separate study has revealed proinflammatory epigenetic changes in men through sex-based comparison of CD4+ T cell DNA methylation in lupus.[29] Notably, significant hypomethylation of the MMP9 gene promoter has been identified as a potential novel biomarker for SLE patients.[30] Furthermore, emerging data suggests an association between intestinal permeability and disease activity, along with DNA methylation changes in lupus patients.[31] The potential utility of RUNX3 methylation levels in PBMC as a biomarker to diagnose disease, predict kidney damage, and assess disease activity in SLE patients is also under investigation.[32]

In this study, we conducted an analysis of mRNA and DNA methylation microarray datasets retrieved from the Gene Expression Omnibus (GEO) database. Our objective was to identify differentially expressed genes (DEGs) between SLE, SLE-LN, and healthy samples. To pinpoint hub genes relevant to both SLE and SLE-LN, we employed weighted gene co-expression network analysis (WGCNA). Subsequently, we delved into the molecular mechanism underlying SLE pathogenesis through functional and

pathway enrichment analysis. Concurrently, we utilized methylation microarray data to identify differentially methylation sites across SLE, SLE-LN and healthy samples. By amalgamating the outcomes of differential gene and differential methylation data analyses, we discerned a series of pivotal hub genes closely linked to the progression of SLE and SLE-LN. In the final phase of our study, we employed reverse transcription polymerase chain reaction (qRT-PCR) to validate the expression of seven genes in the peripheral blood mononuclear cells (PBMCs) from SLE-LN patients and the control subjects. Additionally, we conducted Western blot experiments to verify the protein expression level of two key candidate marker gene in SLE-LN patients and the control group.

DATA AND METHODS

Data sources

Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) of 30 patients with SLE in peripheral blood mononuclear cells (peripheral blood mononuclear cells, Unnormalized Series Matrix File of PBMC whole-genome transcriptome (GSE81622) and DNA methylation microarray (GSE82218), including 15 with LN (SLE LN+, SLE patient with lupus nephritis) and 15 without LN (SLE LN-, SLE patient without lupus nephritis), and 25 normal controls (NC).

Gene co-expression analysis

WGCNA (weighted gene co-expression network analysis) is an analysis method for analyzing gene expression patterns in multiple samples, which can cluster genes with similar expression patterns. The association between modules and specific traits or phenotypes was analyzed. Using R package WGCNA[33] were analysis, select and disease phenotype most relevant function module for subsequent enrichment analysis, genetic variance analysis and define the hub genes.

Gene functional enrichment analysis

Based on the Gene Ontology DATABASE[34] and KEGG PATHWAY DATABASE[35] biochemical pathways of DATABASE, the enrichment of function analysis of the candidate loci are performed. The statistical algorithm (Fisher's exact test) was used to find out which specific functional items a group of genes was most related to. Each item in the analysis results corresponds to a statistical value *P* value to indicate the significance. The smaller the P value is, the greater the relationship between the item and the input gene is. In other words, most of the genes in the group have the corresponding descriptive functions of the entry.

Differential gene screening

Differential expression or differential methylation was calculated with the R package Limma[36] (microarray data). Differentially expressed genes threshold for $|\log2\ FC| > 1.2$ and $P < 0.05$. Difference in methylation site threshold for $|\log2\ FC| > 1.2$ and $P < 0.05$.

Table 1: Primer sequences used in this study (qRT-PCR)

Gene	Primer	Sequence (5′->3′)
ATP8B4	Forward	TAAAAGTCCGCCATGCACTATC
	Reverse	ACTTGTTGTTAGGCACCTCAC
CEACAM1	Forward	TGCTCTGATAGCAGTAGCCCT
	Reverse	TGCCGGTCTTCCCGAAATG
CEBPE	Forward	CTCCGATCTCTTTGCCGTGAA
	Reverse	CCGAAGGTATGTGGAGGGTAG
CYP4F3	Forward	CAACCCCCGAAACGGAATTG
	Reverse	TTCCTCCGAGCTGTGAATCAG
PADI4	Forward	CAGGGGACATTGATCCGTGTG
	Reverse	GGGAGGCGTTGATGCTGAA
SLC2A5	Forward	GAGGCTGACGCTTGTGCTT
	Reverse	CCACGTTGTACCCATACTGGA

Patient and clinical specimens

Twenty-five patients with SLE-LN and 25 healthy volunteers were recruited for the project. Each patient signed an informed consent, and all experiments were conducted in accordance with the guidelines of the Scientific Research and Clinical Trial Ethics Committee of the First Affiliated Hospital of Zhengzhou University, which approved all aspects of this study. We collected PBMC from 25 patients with lupus nephritis and 25 normal fresh samples as controls. All samples were confirmed by laboratory pathology. Three patients and reference samples used as biological repetition in western blot experiments.

qRT-PCR

Using the TRIzol (Invitrogen, United States) to extract total RNA from BMSCs. cDNA was retrieved by PrimeScript™ RT kit using gDNA Eraser. Thereafter, RT-qPCR was performed by SYBR® Premix Ex Taq™ II and Bulk kits (Takara, China). RT-qPCR conditions were one cycle of denaturation at 95°C lasting 30 s and forty cycles of denaturation at 95°C lasting 5 s and annealing at 60°C lasting 34 s. β-actin was used as an internal reference. The primer sequences were as follows: human actin, 5' -cct aga agc att tgc ggt gg-3 '(forward), 5' -gag cta cga gct gcc tga cg-3' (reverse). Quantification was performed using the 2-ΔΔCq method. The primer synthesis design was completed by Sangon Biotech (Shanghai, China). The primers used are listed in Table 1.

Western blot

BMSC were lysed by protein extraction reagent (Thermo Scientific, USA) and protease inhibitors. Proteins were

collected by centrifugation at 12000 g for 15 min at 4°C. Protein concentration was measured using a NanoDrop 2000 spectrophotometer. Twenty µl of protein was separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was sealed through 5% skim milk powder. Then, at 4°C under and targeted CEACAM1 (1:10000, Abcam Inc., Cambridge, UK), SLC2A5 (Abcam #ab36057, UK), beta actin (the latter, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) of a fight. Thereafter, the membrane was passed through the secondary antibody (1:2,000; ab7090; Abcam, USA) incubation 2 h at room temperature. Immunoreactive proteins were visualized by chemiluminescence kits (Beyotime, China), and band densitometry was performed using ImageJ software.

Statistical analysis

Data are expressed as mean ± SD. One-way analysis of variance was used for comparison between the two groups. For qRT-PCR experiments, expression maps and 2-ΔΔCT values were calculated using GraphPad Prism (version 8.0.1), and a *P* value < 0.05 was considered significant. Correlation studies were performed using Spearman's rank correlation coefficient. GraphPad Prism software was used for statistical analysis.

RESULTS

Comprehensive construction of the expression module

In this study, we applied a web-based approach to gain deeper insights into the molecular mechanisms of lupus nephritis. We conducted co-expression analysis using the normalized expression dataset GSE126209. Weighted gene co-expression network analysis (WGCNA) was employed as a systems biology method to unravel patterns of association among genes across different samples. WGCNA enables the identification of gene clusters or modules.[33] Our clustering results based on WGCNA revealed that the control samples formed distinct clusters, demonstrating tight grouping, while it was challenging to discern clear distinctions between systemic lupus erythematosus patients with nephritis (SLE-LN) and those without nephritis (LN) (Figure 2A). Subsequently, following the derivation of a scale-free topological module, we established a correlation threshold of 0.8 and identified an appropriate soft threshold power of 8 (Figure 2B).

Hierarchical clustering was applied to all genes within the co-expression network (Figure 3A), and the resulting cluster map demonstrated that each module exhibited a high degree of independence, with gene expression within each module showing relatively autonomous behavior. Subsequently, we employed dynamic branch cutting to detect a total of 18 modules, each distinguished by a unique color, as per the WGCNA method (Figure 3B). Notably, two substantial modules, denoted as cyan and blue, were observed in the dataset, alongside several smaller modules, underscoring the close interrelation among the expression levels of numerous genes. To further investigate co-expression similarities, all modules, we computed signature genes based on their correlations and organized them into clusters. This analysis roughly grouped the modules into three categories corresponding to control, SLE-LN, SLE (Figure 3C). These findings were corroborated by a heat map based on the adjacency relationship.

Upon delving further into the correlations between the modules and control, SLE-LN, and SLE (Figure 4), several noteworthy findings emerged. Firstly, the MElightgreen module exhibited the most robust positive correlation with Control (0.85, *P* = 1e-16). Interestingly, this same module displayed the most pronounced negative correlation with SLE and SLE-LN, with values of-0.44 (*P* = 8e-04) and -0.51 (*P* = 6e-05) respectively.

Conversely, the MElightcyan module displayed a distinctly different pattern of associations. It showcased the highest positive correlation with SLE-LN, registering at 0.4 (*P* = 0.003), while the correlation with SLE was notably at 0.058. Furthermore, this module exhibited a positive correlation with the control group, with a coefficient of 0.41 (*P* = 0.002).

In addition, the MEgreenyellow module displayed the most substantial positive correlation with SLE measuring at 0.25. However, it is important to note that associated *P* value was 0.06, which did not reach statistical significance (*P* < 0.05). Conversely, this module exhibited a negative correlation with the control group, with a coefficient of -0.38 (*P* = 0.004).

Identification of hub genes

To pinpoint the hub genes within the network, we conducted a thorough examination of gene connectivity degree within the relevant modules associated with lupus nephritis and lupus erythematosus in the co-expression network. We then closely scrutinized these highly connected genes and initially designated them as potential hub genes in the co-expression network (Tables 2 and 3).

Functional enrichment analysis

To conduct functional enrichment and pathway analysis, we opted to focus on modules with a positive correlation to lupus nephritis, such as the light cyan and light green modules, as well as those a negative correlation. Similarly, we selected modules that exhibited a positive association with lupus erythematosus, such as green yellow module, and modules that showed a negative association. As an illustrative example, we will present the functional

Table 2: Statistics of top 20 hub genes in lupus nephritis

Gene	Degrees	Gene	Degrees	Gene	Degrees	Gene	Degree
CTSG	74	COL17A1	72	MMP8	70	SERPINB10	68
CEACAM6	73	SLPI	72	CEACAM1	69	ABCA13	67
OLR1	73	ARG1	71	CEACAM8	69	BPI	67
PCOLCE2	73	ELANE	71	MPO	69	LCN2	67
PRTN3	73	CD24	70	DEFA4	68	TACSTD2	67

Table 3: Lupus top 20 hub gene statistics

Gene	Degrees	Gene	Degree	Gene	Degree	Gene	Degree
ATP6V0D1	78	LOC255809	37	GCHFR	30	LTB4R	23
TRAPPC5	63	NCALD	37	COX8A	26	PLP2	23
PYCARD	57	BRI3	35	CUEDC2	26	RNH1	23
AGTRAP	39	SULT1A3	34	PYHIN1	24	CHMP2A	22
ATP6V0B	39	IMPA2	32	RHOG	24	FCGRT	21

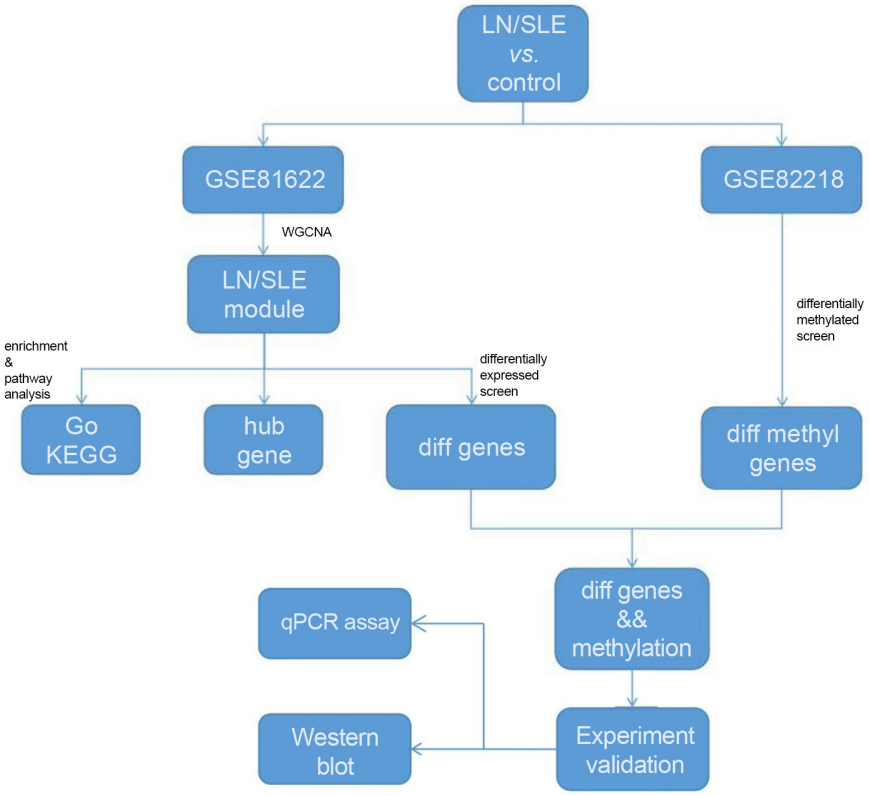

Figure 1: Flow chart of the overall analysis of this study.

enrichment results for genes related to lupus nephritis (Table 4, Table5, Figure 5).

From the gene ontology (GO) enrichment analysis, the top GO terms revealed significant associations with various biological processes, cellular components, and molecular functions. In terms of biological processes (GO-BP terms), the most highly correlated terms includedz

Table 4: The GO-BP enrichment of genes associated with lupus nephritis results (in part)

ID	Term	Count	*P* value	Enrichment score
GO:0016192	vesicle-mediated_ transport	70	2.60 e-21	20.586
GO:0007165	signal_transduction	70	6.27 e-03	2.203
GO:0045321	leukocyte_activation	63	2.22 e-34	33.654
GO:0046903	secretion	62	1.25 e-28	27.903
GO:0032940	secretion_by_cell	61	2.17 e-30	29.664

Table 5: Results of KEGG pathway analysis of lupus nephritis (in part)

ID	Term	Count	*P* value	Enrichment score
hsa04650	Natural_killer_cell_mediated_cytotoxicity	17	2.70 e-13	12.568
hsa04612	Antigen_processing_and_presentation	11	2.32 e-09	8.634
hsa05332	Graft-versus-host_disease	8	2.82 e-08	7.549
hsa04621	NOD-like_receptor_signaling_pathway	8	1.75 e-03	2.758
hsa05150	Staphylococcus_aureus_infection	6	1.11 e-03	2.957

neutrophil_activation, neutrophil_degranulation, and neutrophil_degranulation. neutrophil_activation_involved_ in_immune_response, and neutrophil_mediated_immunity. These processes involved ABCA13 ANXA3, ARG1, ATP8B4, AZU1, BPI, BST1, CAMP, CAT, CCL5, CD63 and CDA gene (Figure 5A and 5B).

In the context of cellular components (GO-CC terms), the module genes were associated with secretory_granule, secretory_vesicle, specific_granule, secretory_granule_ lumen, and cell Mass vesicle lumens (cytoplasmic_vesicle_ lumen), cystic cavities (vesicle_lumen) and cytoplasmic vesicles (cytoplasmic_vesicle), among other relevant components (Figure 5C).

Regarding molecular functions (GO - MF terms), the enriched terms included MHC class I receptor activity, serine peptidase activity, serine-type_endopeptidase_ activity, and serine hydrolase (Figure 5D). Additionally, we assessed the KEGG Pathway score for each sample, with significant associations identified in pathways such as Natural_killer_cell_mediated_cytotoxicity and Antigen Processing and Presentation.

The enrichment analysis highlighted several significant pathways including Antigen_processing_and_presentation, graft-to-host disease, and Staphylococcus_aureus_infection (Figure 5E). These findings underscore the relevance of immune-related genes and pathways, underscoring the for exploring novel immune markers.

Identification of differential mRNA expression

We conducted differential mRNA analysis using a dataset comprising 15 SLE, 15 SLE-LN, and 25 control samples. Our analysis revealed a total of 125 differentially expressed genes (DEGs), in the SLE sample analysis, with 49 genes being up-regulated and 76 genes down-regulated (Figure 6A) Similarly, in the SLE-LN sample analysis, we identified 156 DEGs, including 70 up-regulated and 86 down-regulated genes (Figure 6B).

To gain further insights, we conducted clustering analysis of the top twenty up-regulated and down-regulated genes. This analysis indicated the both SLE and SLE-LN samples could be roughly divided into two clusters based on their gene expression profiles when compared to the control samples. In the SLE samples, the up-regulated genes included CCR1, FPR2, FLJ22662 and CD163 (Figure 6C), while the down-regulated genes included SH2D1B cut gene, KLRF1, KLRB1, CD160 and AUTS2, and others. In the SLE-LN samples, notable up-regulated genes were HP, RNASE3, S100P, CEACAM8, CAMP and DEFA4 were up-regulated, while HDC, FCER1A, KLRC2, KLRC2 and KIR3DL2 were down-regulated (Figure 6D).

Identification of differential methylation sites

For the differential methylation sites analysis, we utilized a dataset consisting of 15 SLE, 15 SLE-LN and 25 control samples. Our analysis revealed a total of 12432 differentially methylated sites in SLE samples, comprising 2260 hypermethylated sites and 10172 hypomethylated sites (Figure 7A). In the SLE-LN samples, we identified 9613 differentially methylated sites, with 4542 being hypermethylated and 5071 being hypomethylated (Figure 7B).

Cluster analysis was performed on the top 20

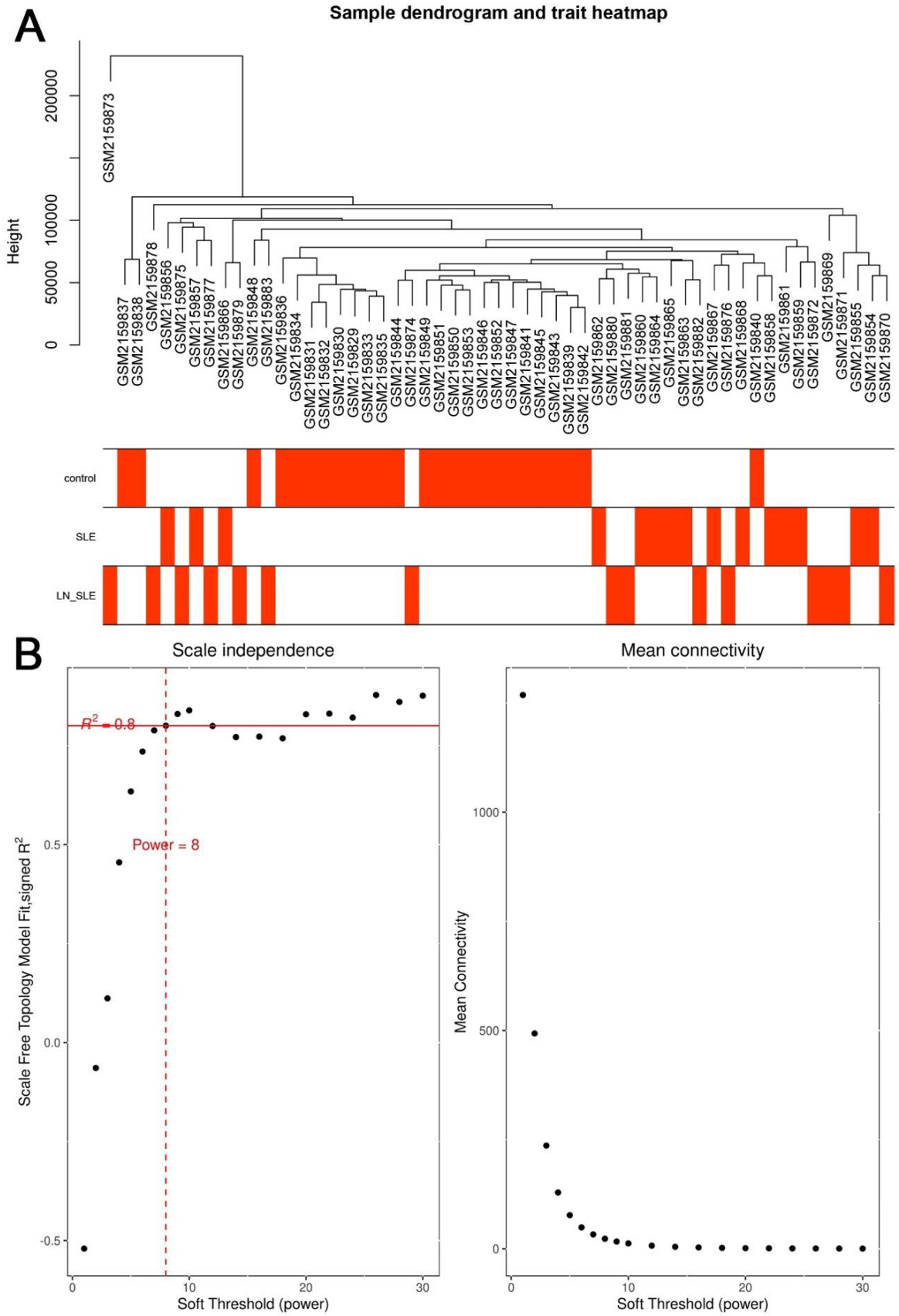

Figure 2: Cluster analysis of co-expression network samples. A. Sample clustering diagram; B. Power value determination map.

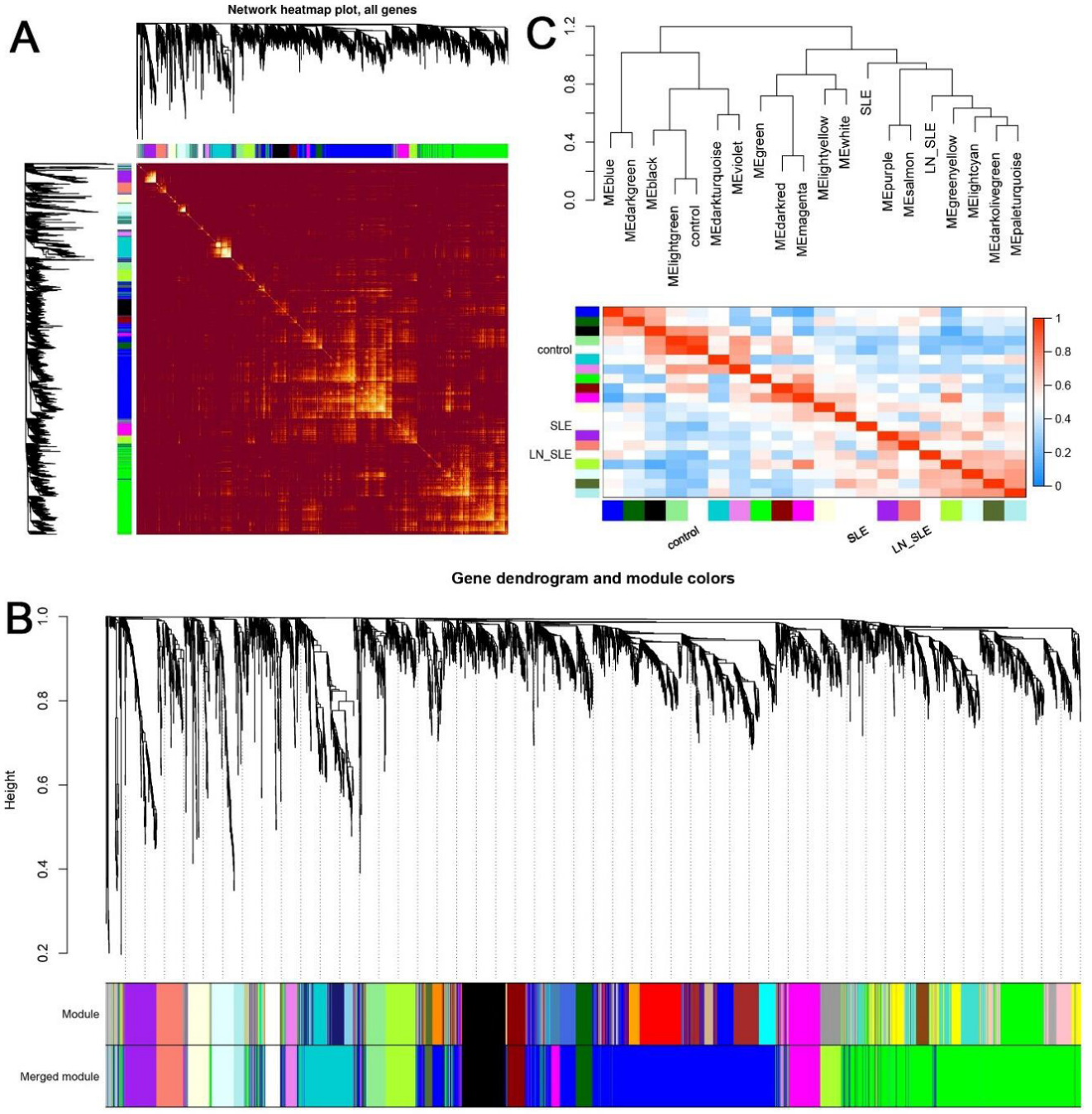

Figure 3: The results were module. A. Gene correlation gene clustering heat map; B. A total of expression module clustering and correlation heat maps of module; C. Gene clustering and corresponding co-expression module map.

hypermethylated and hypomethylating sites, demonstrating that both SLE and SLE-LN samples could be segregated into two clusters based on their DNA methylation profiles. In the SLE samples, hypermethylated sites included cg20064778, cg24565820, cg01454815, cg01558909, cg24844518 and cg20805133, etc. The hypomethylation sites included cg03607951, cg06872964, cg10549986, cg26312951, cg05696877, cg07839457, cg06188083 and cg14864167. In SLE-LN samples, hypermethylation sites included cg22926869, cg14241738, cg06905762, cg10890644, cg13353337 and cg02491947 (Figure 7C), etc. The hypomethylation sites included cg20460697, cg19499452, cg01948202, cg06981309 and cg14864167 (Figure 7D).

Combined analysis of differential genes and differentially methylated genes

We conducted an integration analysis based on the results

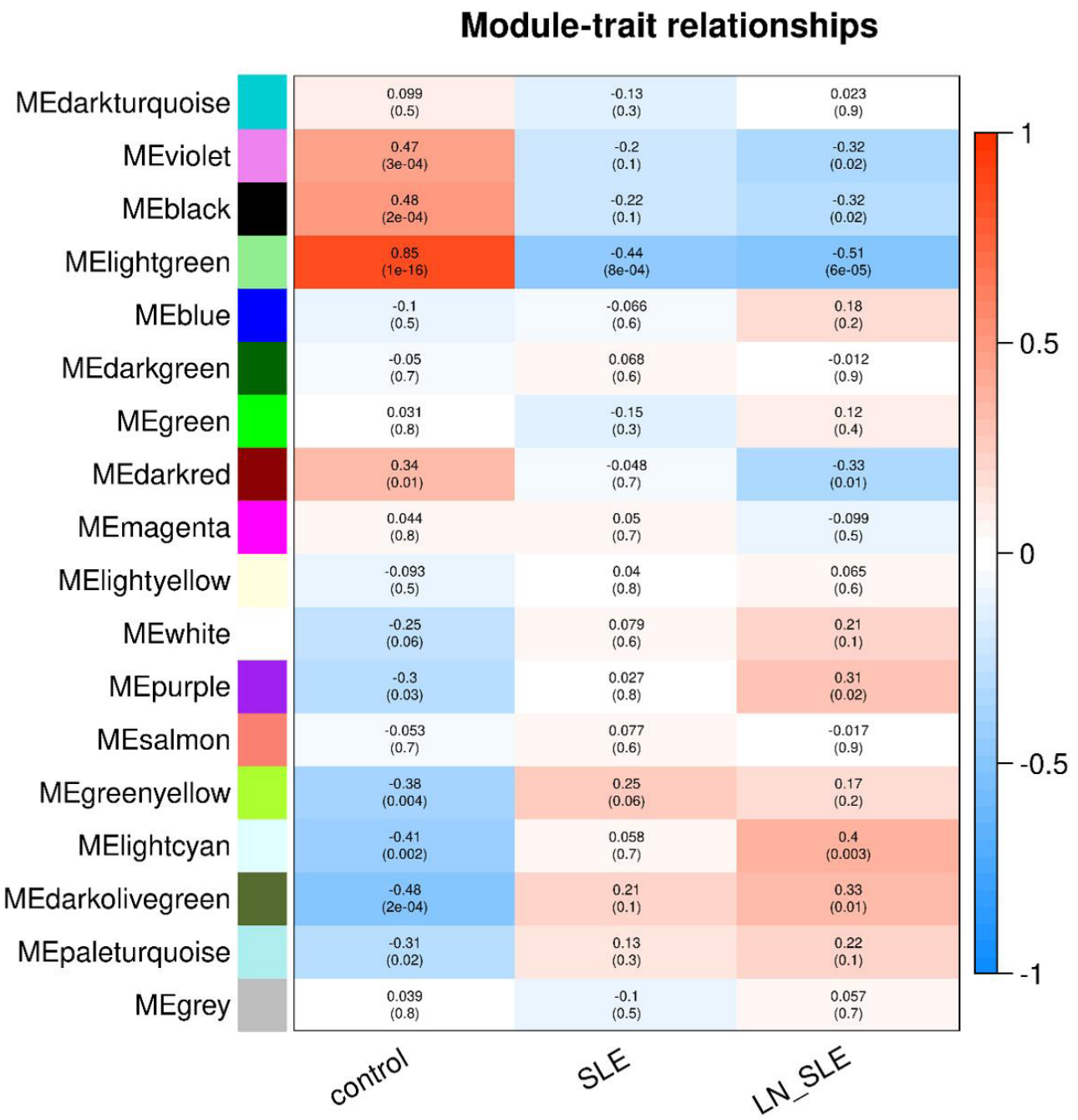

Figure 4: Heatmap of correlation between each expression module and trait. Note: *P* values are in parentheses and correlation coefficients are above the parentheses.

of differential genes and differentially methylation sites, focusing on the intersection between these two sets of genes. The findings revealed: in lupus nephritis, there were 14 genes that overlapped between down-regulated genes and hypermethylated genes (Figure 8A), These genes included AUTS2, CD247, EVL, MATK, NPC1, PRKCH, RAB22A, RHOC, RUNX3, S1PR5, SYTL2, TGFBR3, ZNF573 and ZNF683. Ten lupus nephritis up-regulated genes overlapped with hypomethylated genes (Figure 8B), including ATP8B4, C19orf59, C5orf32, CEACAM1, CEBPE, CYP4F3, LHFPL2, PADI4, RRAGD and SLC2A5. Seventeen lupus erythematosus downregulated genes overlapped with hypermethylated genes (Figure 8C), including CD247, CD7, CD96, CLIC3, CST7, GIMAP7, GZMA, GZMB, IL2RB, MYOM2, NCR3, NLRC3, NPC1, PRF1 PYHIN1, RUNX3 and SYTL2, SLE raised genes with low methylation intersection of 24 genes (Figure 8D), including AGTRAP, ALDH2, ASGR2, C9orf167, CD33,

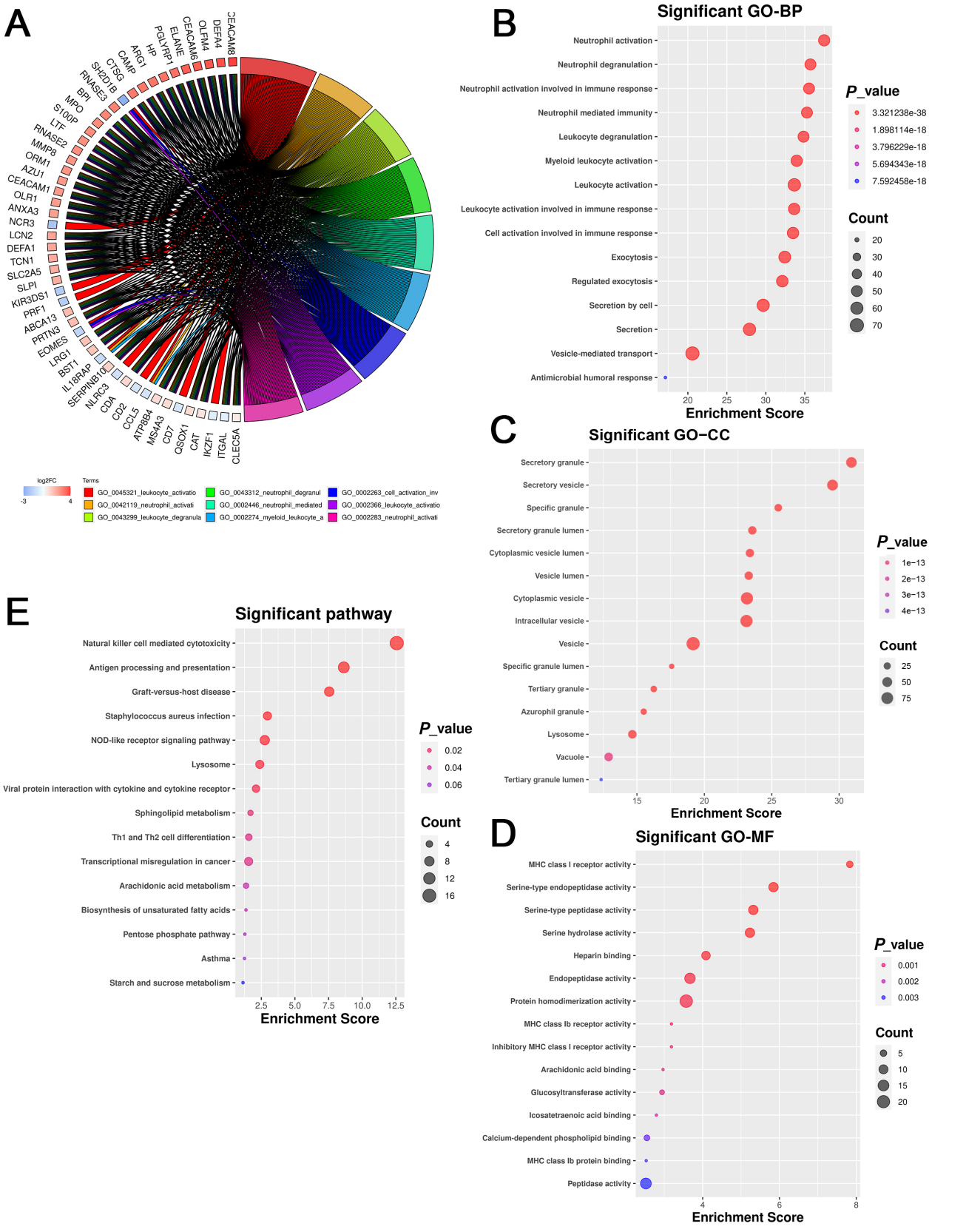

Figure 5: Results of enrichment analysis for Lupus nephritis. A. Enrichment loop of GO-BP top 9 items and top 50 genes in lupus nephritis; B. Bubble plot of the top 15 enrichment entries in GO-BP for lupus nephritis; C. Bubble plot of the top 15 gene enrichment entries of GO-CC in lupus nephritis; D. bubble plot of the top 15 gene enrichment items of GO-MF in lupus nephritis; E: Bubble plot of the top fifteen gene enrichment entries in the lupus nephritis KEGG Pathway.

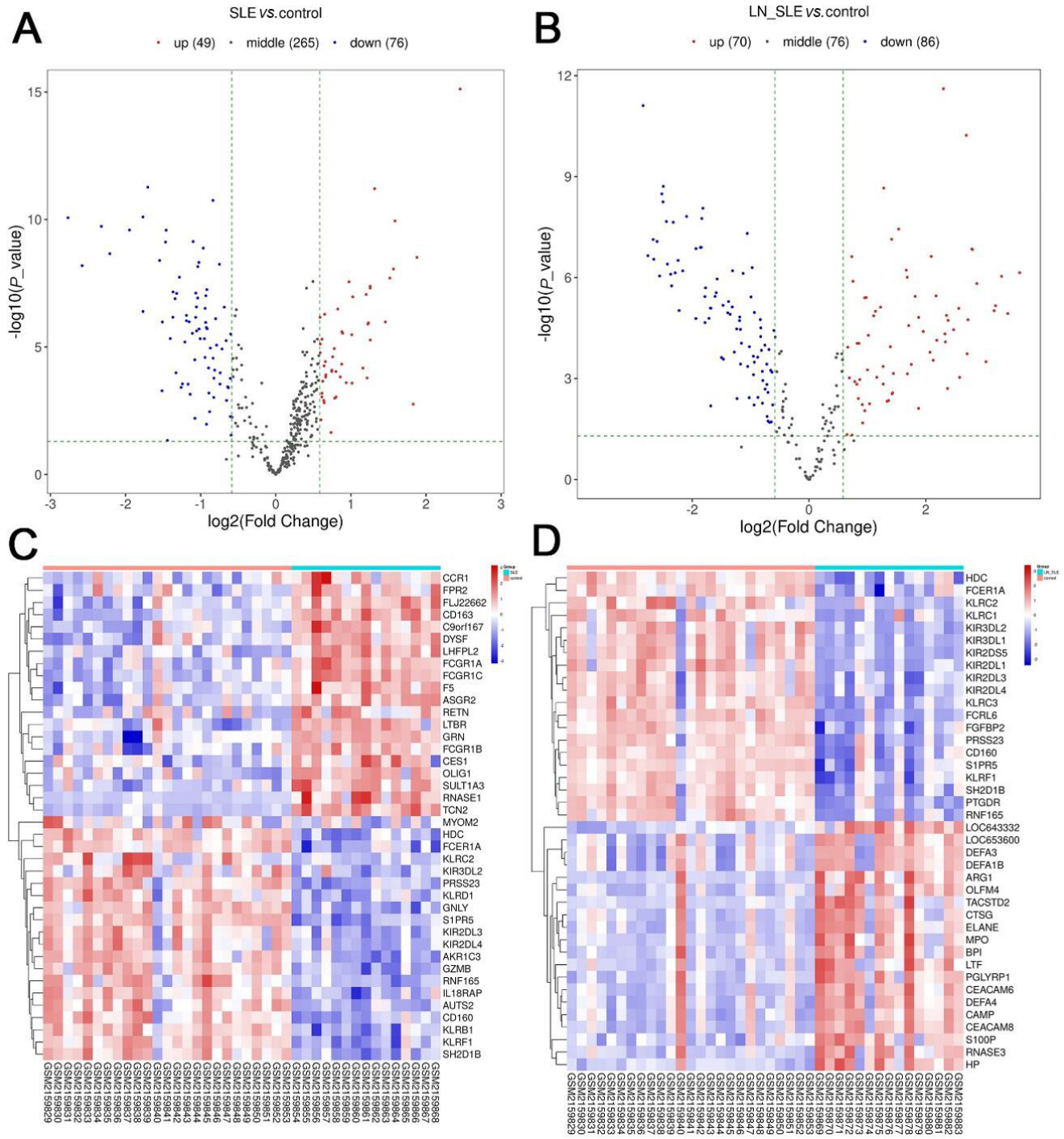

Figure 6: Differential gene results in lupus erythematosus and lupus nephritis. A. Lupus erythematosus differentially expressed genes volcano diagram; B. Lupus nephritis differentially expressed genes volcano map; C. Heat map of top 20 differentially expressed genes in lupus erythematosus; D. Heat map of top 20 differentially expressed genes in lupus nephritis.

CES1, DYSF, FPR2, GRN, HK3, LHFPL2, LTBR, MAFB, NCF1C, NCF2, NLRP12, OLIG1, OSCAR, PLAUR, PNPLA2, QPCT, RRP12, SIRPA, and SORT1.

Verification of candidate marker genes for lupus nephritisTo validate the conclusions drawn from our analysis, we conducted qRT-PCR experiments on seven selected genes—ATP8B4, CEACAM1, CEBPE, CYP4F3, PADI4, RRAGD and SLC2A5—taken from the intersection of up-regulated genes in lupus nephritis. The results showed that CEACAM1 exhibited the most significant increase in expression, followed by CYP4F3,

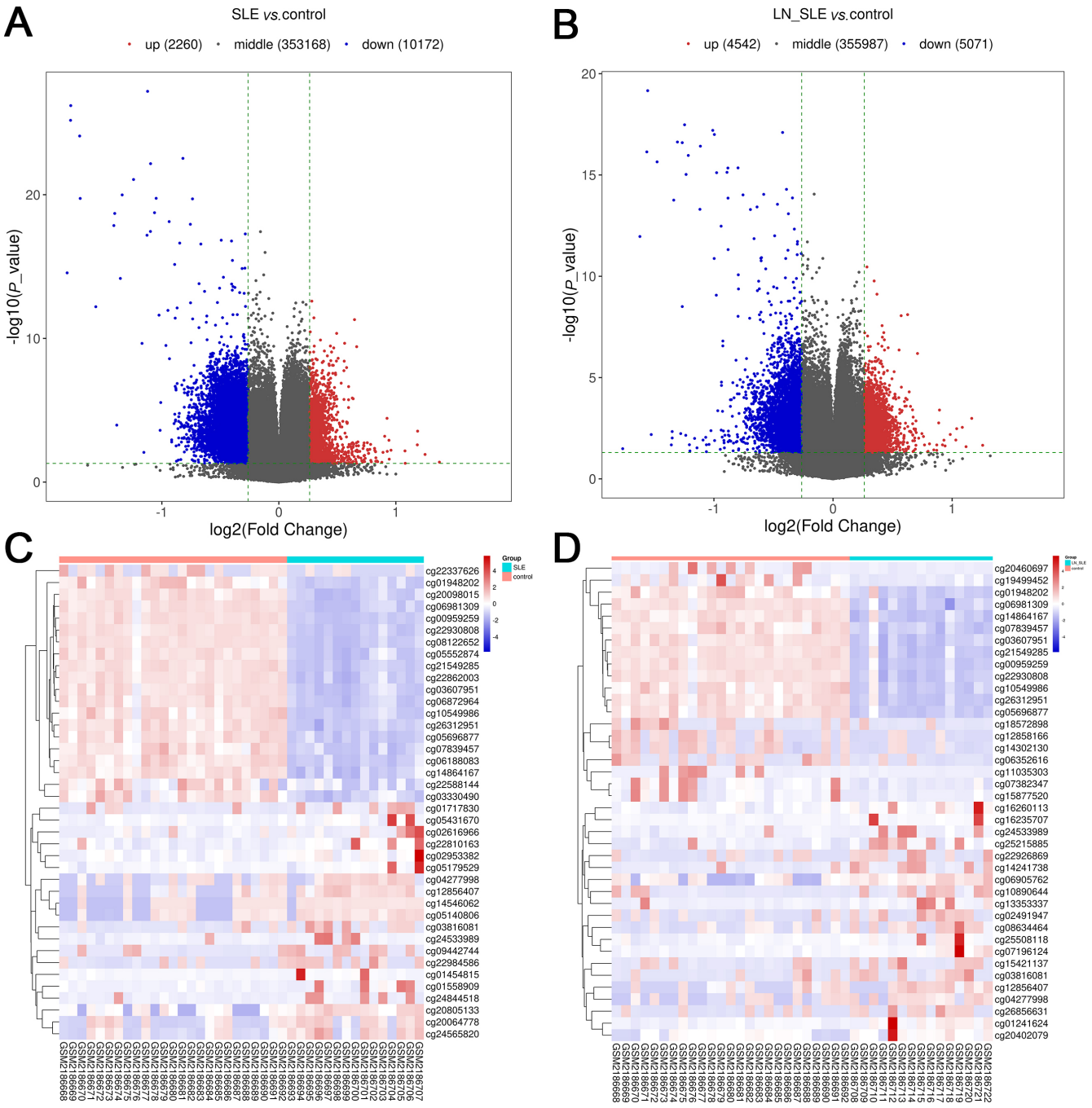

Figure 7: Results of differential methylation sites in lupus erythematosus and lupus nephritis. A. Volcano plot of top 20 differentially methylated sites in lupus erythematosus; B. Volcano plot of top 20 differentially methylated sites in lupus nephritis; C. Heat map of top 20 differentially methylated sites in lupus erythematosus; D. Heat map of top 20 differential methylation sites in lupus nephritis.

PADI4, and SLC2A5, while ATP8B4 and CEBPE raised expression was not significant. Furthermore, we examined the protein expression levels of CEACAM1 and SLC2A5. Similarly, CEACAM1 was upregulated most significantly, while SLC2A5 was upregulated. These findings provide strong confirmation of the reliability of our analysis and suggested that genes such as CEACAM1 and SLC2A5 may serve as potential markers for lupus nephritis (Figure 9).

DISCUSSION

SLE is a chronic autoimmune disease with complex clinical manifestations, often leading to systemic damage, especially in the form of SLE-LN. Currently, there is no effective cure for SLE, and patients often require long-term or even lifelong medication, significantly impacting their quality of life. Statistically, there are approximately 150 cases of

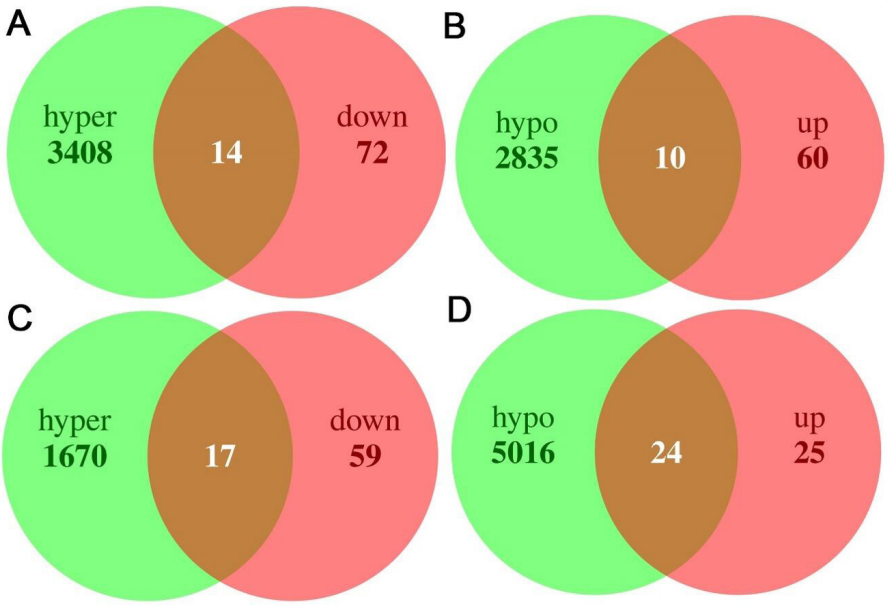

Figure 8: Venn plot of differentially expressed genes versus genes corresponding to differentially methylated sites. A. Venn diagram of down-regulated and hypermethylated genes in lupus nephritis; B. Venn diagram of up-regulated and hypomethylated genes in lupus nephritis; C. Venn diagram of down-regulated and hypermethylated genes in lupus erythematosus; D. Venn plot of up-regulated versus hypomethylated genes in lupus erythematosus.

SLE per 100000 people, and 15% of SLE patients develop renal failure or succumb to the disease within 5 years.[37] The development of SLE is influenced by various factors, including genetics, the environment, epigenetics, hormones and more. However, the exact underlying mechanisms of SLE remains unclear. Therefore, further investigation into the pathogenesis, particularly the identification of effective biomarkers for early diagnosis and treatment, is crucial for enhancing the quality of life for patients.

In this study, we utilized the expression microarray dataset GSE81622 to identify key genes, particularly CEACAM1 and SLC2A5, for constructing diagnostic gene models for SLE. Notably, CEACAM1 was also identified as a hub gene within the WGCNA module. CEACAM1 and SLC2A5 exhibited high expression levels and hypomethylation in lupus nephritis, and their gene and protein expression were confirmed in lupus nephritis patients. Moreover, in a parallel analysis of patients with systemic lupus erythematosus without nephritis, CEACAM1 and SLC2A5 displayed hypomethylation, even though they did not exhibit elevated expression levels. Additionally, existing literature research has reported elevated CEACAM1 expression in the peripheral blood mononuclear cells (PBMCs) of SLE patients. Inhibition of CEACAM1 expression was found to hinder the differentiation of CD4+ CD25+ regulatory T cells (Tregs) and promote autoimmunity, corroborating the reliability of our analysis.[38] It also confirmed our analysis of reliability. In addition, CEACAM1 was selected as one of the core shared genes in an identification of common genetic features and molecular pathways in systemic lupus erythematosus and diffuse large B-cell lymphoma. These genes showed strong associations with inflammatory and immune response pathways. These findings could potentially offer new biomarkers and therapeutic targets for SLE.[39]

Recent research in the field of epigenetic has increasingly highlighted the significance of gene methylation as a critical mechanism in the onset and progression of diseases. In a study of how DNA methylation affects clinical heterogeneity in SLE, the authors found that differential methylation of genes that regulate tissue hypoxia response and interferon-mediated signaling is associated with lupus nephritis in women with SLE, suggesting that epigenome-wide association studies can help identify genomic differences behind clinical heterogeneity in SLE.[40] CpG islands are defined as DNA fragments ranging from 500 and 1500 base pairs in length with a CG base to GC base ratio exceeding 0.6. Typically, CpG islands are situated in gene promoter regions and encompass the 5' end of transcripts. From an evolutionary standpoint, CpG islands have evolved to be considered functional and crucial components in defining and regulating vertebrate promoter regions. CPGS islands are well-known for their close association with aberrant DNA methylation and play a role in determining active or potentially active promoter regions.[41] Methylation levels can regulate gene expression. High promoter methylation often results in decreased gene expression, while low promoter methylation is linked to increased

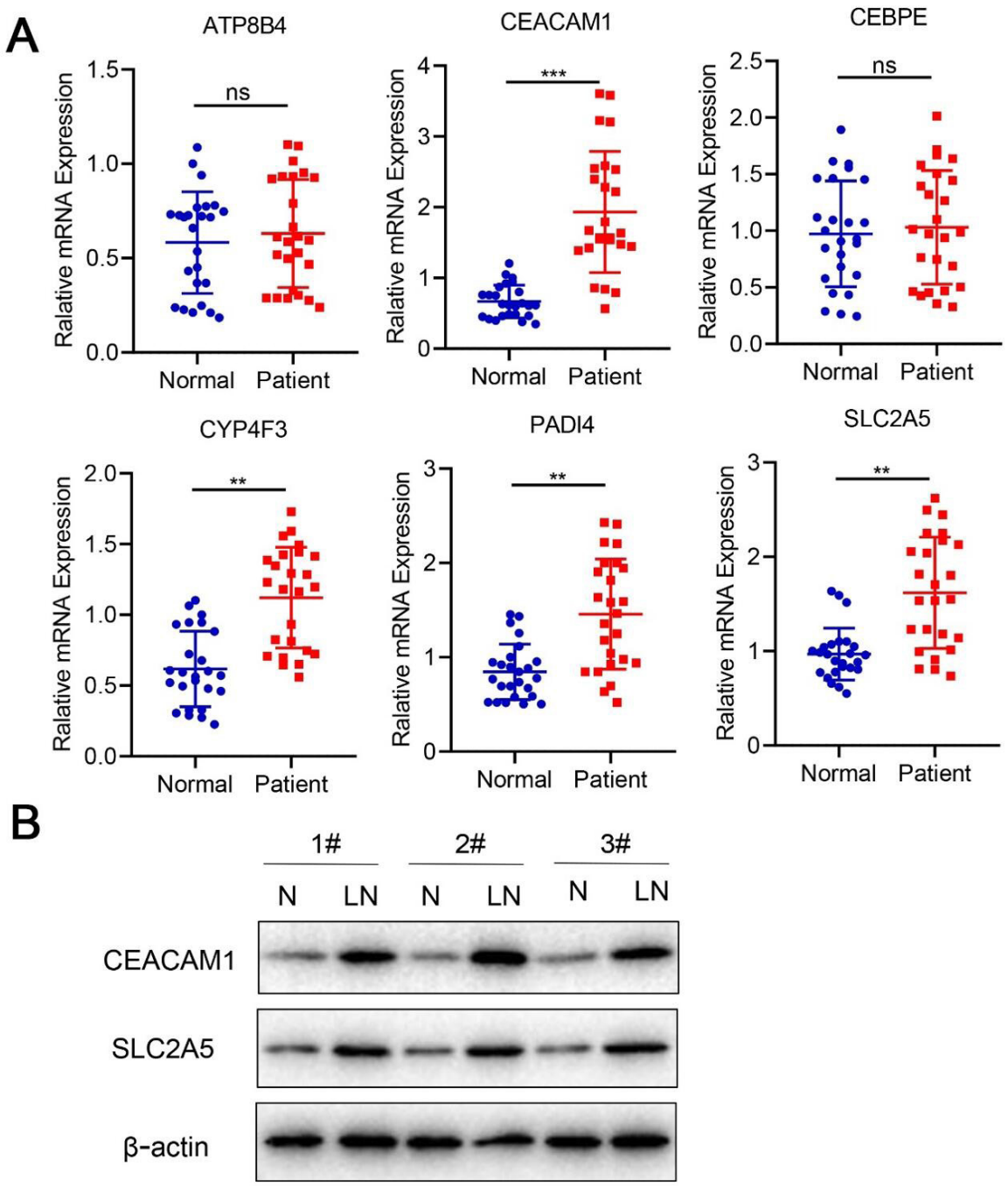

Figure 9: Experimental validation of candidate marker genes for lupus nephritis. A. qRT-PCR experiments to verify the expression of key hub genes between SLE-LN and healthy samples; B.Western blot experiments to verify protein expression of key hub genes between SLE-LN and healthy samples. **$P < 0.01$ and ***$P < 0.001$.

gene expression.[42] Moreover, hypomethylation of CpG dinucleotides in specific genomic regions, can lead to loss of imprinting, reactivation of transposable elements, and ultimately, chromosomal instability.[43] Prior investigations have proposed that the gradual process of DNA methylation plays a functional role in the pathogenesis of SLE. Within SLE patients, specific autoimmune-related genes, such as ITGAL, TNFSF7, PRF1, are found to display hypomethylation within the promoter regions of CD4+ T cells.[44,45] Studies have confirmed that transcriptomic and epigenetic alterations in dendritic cells correspond with chronic kidney disease in lupus nephritis, and these findings present dendritic cell alterations that may reflect renal involvement in SLE, laying foundations for new strategy of diagnosis and monitoring of LN patients, omitting invasive kidney biopsies.[46] Furthermore, numerous comprehensive assessments of DNA methylation across the genome have pinpointed a multitude of genes that exhibit differential

methylation patterns characterized by hypomethylation in SLE patients.[47,48] Nevertheless, the specific methylation status of CEACAM1 and SLC2A5, particularly in relation to SLE-LN, remains unexplored. CEACAM1, also known as CD66a, is a member of the carcinoembryonic antigen family and is widely expressed in various cell types.[49] It plays a crucial role in regulating intercellular adhesion, cell growth, and differentiation. When expressed on the cell membrane, it interacts with integrins or extracellular matrix proteins to modulate the immune response. As an ITIM type 1 membrane protein, its primary function in lymphocytes is to generate inhibitory signals,[50] although its exact inhibitory role has not been fully elucidated. Previous studies have suggested that CEACAM1 can bind to TIM-3 in cells to regulate TIM-3 mediated tolerance and depletion.[51] In vitro studies have shown that CEACAM1 is co-expressed with TIM-3 in activated T cells and may contribute to T cell tolerance.[52] The interaction between CEACAM1 and TIM-3 is complex, requiring further comprehensive investigation into the underlying molecular mechanisms related to changes in CEACAM1 expression in LN and its intracellular signal transduction. SLC2A5, also known as glucose transporter 5 (GLUT5), is a brush-border protein involved in fructose absorption from the lumen.[53] Its expression increases during the transition from the crypt to the villus. Previous research has indicated that histone H3 methylation at lysine 4 on the SLC2A5 gene in intestinal Caco-2 cells influences SLC2A5 expression.[54] Another study reported that S100P contributes to promoter demethylation and transcriptional activation of SLC2A5 by integrating into specific regions of SLC2A5 promoter, thereby reducing its methylation level and activating SLC2A5 transcription.[55] Future investigations will focus on verifying this relationship by studying how CEACAM1 and SLC2A5 expressions relate to interacting molecules or immune cells during LN inflammation.

Our research, which utilizes differential expression and methylation analyses, not only contributes valuable insights into cancer research but also provides a reference point for further investigations, especially concerning DNA methylation level their impact on gene methylation and hypomethylation patterns. The study, however, is subject to certain limitations that should be considered.

Firstly, the sample size of the GSE81622 dataset was relatively small, and we relied on the analysis of a single dataset. Consequently, the result may have some deviation. Secondly, our observations were primarily focused on peripheral blood mononuclear cells (PBMC). PBMCs comprise a mixture of various cell types, and it would be beneficial for future research to employ diverse experimental methods to validate and expand upon our findings within different cell types. Thirdly, the sample size in our experimental validation was also limited, which may affect the generalizability of our results.

Lastly, we did not perform experimental validation of methylation changes, emphasizing the need for further investigation to validate the robustness and reliability of our study's findings in subsequent research endeavors.

CONCLUSION

In summary, our findings indicate that the CEACAM1 and SLC2A5 gene, characterized by their heightened expression levels in the context of lupus nephritis and concurrent low levels of DNA methylation, hold promise as potential diagnostic markers for SLE-LN.

Author Contributions

Liu Z and Xia Z designed the experiments; Zhao Z and Xie J performed the research; Pan S and Liu D contributed essential reagents or tools; Liu Z and Liu F analyzed the data; Liu Z and Liu F wrote the paper.

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Ethical Approval

The study has been approved by the review committee of the First Affiliated Hospital of Zhengzhou University.

Informed Consent

All participants provided their informed consents.

Conflict of Interests

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.

Data Availability Statement

Additional data will be provided upon reasonable request by contacting the corresponding author.

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