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Comprehensive analysis of eccDNA characteristics and associated genes expression in peripheral blood of ASLE and ISLE patients

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ABSTRACT

To explore SLE staging markers, we analyzed eccDNA in plasma using circular sequencing, comparing healthy controls (HC), active SLE (ASLE), and inactive SLE (ISLE) patients. We found higher eccDNA levels and lower GC content in ASLE and ISLE compared to healthy controls, with a negative correlation between GC content and anti-daDNA, C3, and C4 levels in SLE and HC samples. Differential expression of exon-derived eccGenes in ASLE and ISLE suggests their role in SLE development, with KEGG analysis showing enrichment in SLE-related pathways for these differentially expressed genes. By protein–protein interactions network analysis we found 9 exon-derived eccGenes that were significantly differentially expressed and scored high in both ISLE-HC and ASLE-ISLE as diagnostic criteria for differentiating different disease stages of SLE. In conclusion, the present study reveals that eccDNA length GC content as well as chromosomal distribution in ASLE, ISLE and HC suggests that with eccDNA is associated with the creation of SLE, suggesting GC count of eccDNA as a diagnostic marker for systemic lupus erythematosus. Significant changes in the abundance of eccDNA-related genes from exons such as SOS1, GAD2, BCL11B, PPT1, and GCNT3 were observed in ISLE as compared to ASLE and HC groups and were significantly correlated with SLEDAI-2K. This suggests that these exon-derived eccGenes may play a role in the development and progression of the disease. Consequently, the abundance levels of these exon-derived eccGenes could potentially assist in distinguishing different stages of SLE, beyond a confirmed diagnosis, thus serving as possible biomarkers for the condition.

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Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder marked by the production of numerous autoantibodies, leading to varied clinical manifestations such as skin, mucocutaneous, joint, muscle, kidney, and cardiovascular involvement [1,2]. It affects approximately 5.14 per 100,000 individuals annually worldwide, with an estimated prevalence of 43.70 per 100,000. Each year, about 400,000 new cases are diagnosed, contributing to a prevalent population of 3.41 million individuals globally [3]. SLE can be divided into active and inactive phases, assessed by the disease activity index. Active systemic lupus erythematosus (ASLE) represents a more severe phase, characterized by an overly aggressive immune response that targets the body's own tissues, leading to widespread organ and systemic damage. Conversely, inactive systemic lupus erythematosus (ISLE) denotes a phase of relative stability or remission, often displaying milder symptoms or none at all [4]. The SLE Disease Activity Index 2000 (SLEDAI-2K) is commonly used in clinical settings and research [5]; however, it has notable limitations, such as failing to

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reflect changes within specific organ systems and its sometimes disproportionate severity weighting. Accurately assessing SLE activity is crucial for guiding treatment decisions and improving patient prognoses [6].

EccDNA(extrachromosomal circular DNA), comprises circular DNA molecules within the cell nucleus, known for their stable double-stranded structure [7]. This stability offers potential use as a blood biomarker, potentially addressing the limitations of the SLEDAI-2K. EccDNA varies significantly in size, ranging from a few hundred base pairs (bp) to several megabases (Mb) [8–11]. These molecules can be categorized into several classes based on size and sequence characteristics: small polydisperse circular DNA (spcDNA), episomes, microDNA, telomeric circular DNA, double minutes (DM), and extrachromosomal DNA (ecDNA). Additionally, eccDNA has been explored as a biomarker in various cancers, aiding in diagnosis and prognosis prediction [12]. The role of eccDNA in autoimmune diseases is becoming increasingly apparent. Studies have shown that the eccDNA profiles in autoimmune conditions like rheumatoid arthritis (RA) and type 2 diabetes differ significantly from those in healthy individuals, not only in terms of length range and genomic distribution but also in functionality and biological processes [13,14]. However, there is a notable gap in research regarding eccDNA in active versus inactive SLE. Studies in SLE have shown that specific gene CF-eccDNA profiles can all be biomarkers of SLE activity [15]. And the results of Gerovska et al. pointed out that between the genes of the SLE mouse model Dnase1l3 and the human single-gene SLE partner DNASE1L3 defects both showed gene cf-eccDNA profiles with a large number of genes specific to the disease group, confirming the promise of gene cf-eccDNA in the study of SLE [16].

This study aims to bridge this gap by examining the gene distribution and length range of eccDNA in the peripheral blood of patients with both active and resting SLE. By comparing these findings with a healthy control group, we intend to explore the potential functions or mechanisms of eccDNA in SLE. This investigation could provide novel serum markers for distinguishing between clinical ASLE and ISLE, enhancing diagnostic accuracy and potentially guiding therapeutic interventions.

Materials and methods

Clinical sample collection

This study was approved by the Ethics Committee of Shenzhen People's Hospital (Ethics Committee No:LL-KY-20220322). In this study, 43 cases of SLE and 11 cases of blood samples from healthy people were collected from October 2018 to October 2021 in Shenzhen People's Hospital [17]. Participants included patients diagnosed with SLE according to the 2019 EULAR/ACR classification criteria, Patients have signed an informed consent form The severity of the disease was assessed using the SLEDAI-2K, with the sample comprising 23 patients classified as having inactive SLE (SLEDAI ≤4) and 20 patients identified as having active SLE (SLEDAI >4). All patients were above 18 years of age. Exclusion criteria for this group included the presence of other autoimmune diseases, renal disorders, acute or chronic infections, severe comorbidities, or malignant tumors. Additionally, blood samples were collected from 11 healthy individuals undergoing routine physical examinations. These healthy control (HC) volunteers were age and sex-matched to the SLE patients and screened to ensure they were free from rheumatic immune diseases, renal disorders, infections, cancer, and other significant medical conditions. The primary clinical characteristics of the participants has been presented in Supplementary Table 1.

Plasma extraction, eccDNA extraction and library construction

Vacuum blood collection needle to collect 5–10 ml of venous blood, add EDTA anticoagulation tube centrifuged at 1200 rpm for 5 min; take the supernatant to 15 mL centrifuge tube centrifuged again, centrifuged at 3000 rpm for 5 min; take the supernatant that is the plasma of the samples to the 2.0 mL EP tubes, frozen and stored at −80°C in the refrigerator. We purchased the MGIEasy Circulating DNA Extraction Kit to extract total cell free DNA(cfDNA). For linear cfDNA removal of the total cfDNA obtained, we used the method of Plasmid-Safe ATP-Dependent DNase (PSD

10,000 U/mL, Epicentre) enzymatic digestion in a 50 μL reaction system at 37°C for a duration of 16 hours. Looped cfDNA was added to 100 μL of VAHTS® DNA Clean Beads (Vazyme), while, after that, it was put into a magnetic rack (Invitrogen) (Vazyme) to obtain the magnetic bead-purified looped cfDNA, and finally, we rolled-looped the enriched looped DNA for amplification by RCA [18]. The RCA products were sonicated using a Covaris sonicator to obtain DNA fragments predominantly within the 300–500 bp range. For each sample, 80 ng of these DNA fragments were utilized for library preparation. The library construction was performed using the MGIEasy DNA Library Preparation Kit (MGI-BGI), which included steps of end repair, A-tailing, and adapter ligation.To ensure quality control, the size distribution and concentration of the libraries were precisely measured using a Bioanalyzer 2100 (Agilent). Finally, sequencing was conducted on the MGI DNBSEQ-T7 platform, employing a read length of 150 bp in a paired-end configuration.

Preprocessing of raw sequencing data

The raw sequencing data underwent a rigorous quality control process using FastQC (v0.11.3) and Fastp (v0.21.0) to ensure high-quality reads. Comprehensive sample quality metrics were compiled using MultiQC (v1.10.1). Subsequently, the high-quality, filtered reads were aligned to the human reference genome (GRCh38 December 2013, UCSC) utilizing the BWA-MEM algorithm [19]. For the precise identification of eccDNA regions, we employed Circle-Map (v1.1.4), which analyzes the aligned data to detect eccDNA [20]. Only eccDNA candidates exhibiting two or more split reads were selected for detailed further analysis [21].

Base sequence analysis on both sides of the eccDNA junction

BedTools software (v2.27.1) [22] was used to extract the 10 bp upstream and downstream base sequences of each eccDNA junction and visualized by the R package ggseqlogo. In order to compare the number of eccDNAs generated by different genes, we performed the following algorithmic steps: ① the number of eccDNAs/the length of genes was normalized, ②the result of ①/the total number of reads obtained in the sample, to obtain the eccGene counts per million reads mapped, abbreviated as EPM; ③ finally, the EPM of an eccGene was divided by the total EPM of all genes in the sample to finally obtain the normalized eccGene abundance. EPM); ④Finally, divide the EPM of an eccGene by the total EPM of all genes in the sample to get the normalized eccGene abundance [20,23].

Functional analysis

Differential eccGenes between the ISLE and HC groups, ASLE and HC groups, and ASLE and ISLE groups were identified using the limma package, employing the Robust Multi-array Average (RMA) algorithm. The results of these analyses were visually represented through volcano plots and heatmaps, specifically highlighting the differential eccGenes.The criteria for selecting differential eccGenes were stringent: a p-value of less than 0.01 and an absolute log2 fold change (Log2FC) greater than 1. This ensured the relevance and significance of the genes identified for further study.For the biological process and pathway enrichment analysis, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were utilized. These analyses were conducted using the 'clusterProfiler' package in R software, which facilitates detailed examination of the biological functions and pathways involved [24]. Visualization and further analysis of the data were achieved using the ggplot2 package, enhancing the interpretative clarity of the results.

Protein–protein interaction (PPI) analysis

The protein–protein interactions among the relevant differentially expressed genes were investigated using the STRING database, selecting for an interaction score greater than 0.4 (http://string-db.org/) [25]. The data gleaned from STRING were subsequently imported into Cytoscape (version 3.9.0) for detailed visualization of the PPI network.

Statistical analysis

All statistical analyses were conducted using R software, version 4.3.2. To compare differences

among the three groups, the Kruskal-Wallis test was utilized, providing a robust method for assessing statistical significance in non-parametric data. Additionally, correlation analyses were performed using the Pearson correlation test to evaluate relationships between variables. A p-value threshold of less than 0.05 was established as the criterion for statistical significance.

Result

Characterization of eccDNA in the plasma of ASLE and ISLE patients

In order to find clinically specific markers for differentiating the different stages of SLE, We extracted eccDNA from the plasma of 11 healthy individuals (control group), 23 patients with ISLE stage (ISLE group), and 20 patients with ASLE stage(ASLE group). We then sequenced and analyzed these samples using circle-seq (Figure 1a). The analysis revealed that the ASLE group had the highest amount of eccDNA among the three groups. The ISLE group also exhibited higher eccDNA levels compared to the healthy control group, but the differences were not statistically significant (Figure 1b).GC content of DNA is related to its stability and function, and it is evolutionarily conserved. Previous studies have indicated that high GC content is a common feature of eccDNA, with tissues, cell lines, and urinary eccDNA showing enrichment in high GC content regions [21]. In our study, the healthy control group exhibited the most abundant GC content in eccDNA. In contrast, both the ISLE and ASLE groups had significantly lower GC content compared to the control group, though there was no difference between the ISLE and ASLE groups (Figure 1c). Based on this finding, we hypothesize that the GC content may aid in distinguishing between SLE and HCgroups. To further investigate this, we conducted a detailed analysis of the correlation between GC content and levels of AntidaDNA, C3, and C4 in SLE and HC samples. The results indicated a negative correlation between GC content and these markers, which was statistically significant (Figures 1e-g). Therefore, we propose that GC content could potentially serve as a diagnostic biomarker for SLE. Furthermore, we explored the potential of GC content to differentiate between ISLE and ASLE. Through correlation analysis with the SLE-DAI2K, we found that there was neither a significant difference nor a significant correlation in GC content between ISLE and ASLE (supplementary Figure S1). This suggests that GC content is not effective in distinguishing between ISLE and ASLE

The distribution of cccDNA lengths in ASLE, ISLE and healthy controls showed differences, with eccDNA lengths ranging from 0.01 kb to 1,000 kb. The majority of cccDNAs ranged in length from 0.01 kb to 500 kb (Figure 1d). Consistent eccDNA, defined as Shared eccDNA genes between ASLE, ISLE and healthy controls [26], was detected in 490, 474, and 185 instances in the ASLE, ISLE, and healthy control groups, respectively. This suggests that the chromosomal distribution of eccDNAs in both the ASLE and ISLE groups is similar (Figure 1e).

It has been shown that eccDNA consists of various original sequences such as DNA, low-complexity regions, rolling circle (RC) sequences, retroposons, satellite DNA, small cytoplasmic RNA (scRNA), RNA, signal recognition particle RNA (srpRNA), unknown sequences, ribosomal RNA (rRNA), transfer RNA (tRNA), and small nuclear RNA (snRNA). These eccDNAs are more likely to form in repetitive satellite elements and the 5S rDNA eccDNA [8]. In our study, we found that except for LTRs, simple repeats, long interspersed nuclear elements (LINEs), and short interspersed nuclear elements (SINEs), most of the aforementioned eccDNA constituent sequences showed no significant differences among the three groups (Figure 1f). Among those sequences with significant differences, simple repeats and SINEs exhibited the most notable variations among the three groups.

Frequency of eccDNA genomic distribution and its correlation with SINE region in ASLE and ISLE

eccDNA originates from the 23 pairs of chromosomes, and its formation mechanisms are classified into four categories: 1) the breakage-fusion-bridge (BFB) cycle [27]; 2) the chromothripsis model [28]; 3) the translocation-deletion-amplification model [29]; and 4) the episome model [30]. We examined the distribution

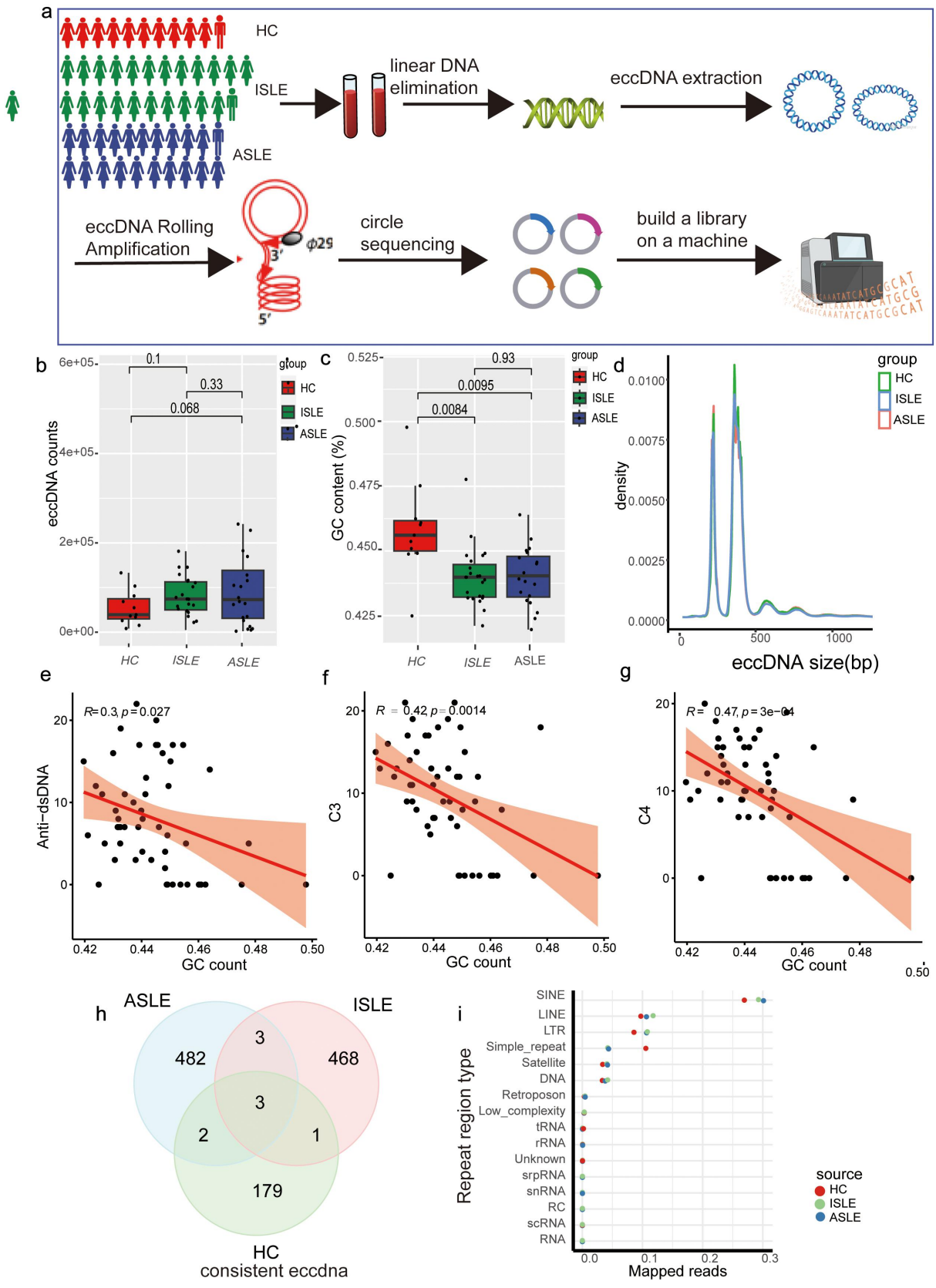

Figure 1. Characterization of eccDNA in the plasma of ASLE and ISLE patients : (a) Schematic representation of eccDNA in ASLE, ISLE and HC blood from extraction, enrichment and sequencing procedures; (b) Counts of eccDNA in ASLE, ISLE and HC; P-value is a statistic that indicates the probability of an observed statistical result (or a more extreme result) occurring if the null hypothesis is true, with a P-value <0.05 the result is usually considered statistically significant; the eccDNA count involves an assessment of the

and frequency of eccDNA per chromosome in the ASLE, ISLE, and HC groups.In the HC group, the eccDNA per megabase (Mb) on each chromosome was lower compared to the other two disease groups. The average frequency of eccDNA per Mb across most chromosomes was around 150, except for chromosome 13, where the frequency was much lower, approximately 100 per Mb. In contrast, chromosomes 17, 19, and 20 showed higher distributions of eccDNA, with frequencies around 100 per Mb. The differences in eccDNA distribution across chromosomes were statistically significant among the three groups (Figure 2a,b). 'Motif' (motif) usually refers to a specific sequence pattern that is repeated in a series of DNA sequences. In the identification of eccDNA sequence motifs, we focused on DNA sequences 10 bp upstream to 10 bp downstream of eccDNA start/end sites (junctions), which are start and end positions that may be related to the formation mechanism of eccDNA. We found no significant differences in the base composition of DNA sequences 10 bp upstream to 10 bp downstream of the eccDNA start/end sites (junctions) in the ASLE, ISLE and HC groups (Supplementary Figure S2A-2C).

In addition, since the reads of the SINE region in the HC group were fewer than those in the two disease groups, we observed significant differences in the number of reads in the SINE region across different chromosomes. For example, SINE reads were almost zero on chromosome Y. Furthermore, we found a positive correlation between the number of eccDNAs per Mb and the number of Alu elements per Mb in the HC, ISLE, and ASLE groups. This positive correlation was particularly significant in the ASLE group (Figure 2c-e).

Exon origin of eccGenes abundance differences and their biological significance between ASLE and HC

To investigate the key genes and significant biological functions involved in the pathogenesis and progression of SLE, we performed differential eccgene abundance analyses in ASLE, ISLE, and HC, and further analyzed the relevant biological functions of these differentially abundance genes.

In the comparison between ASLE and ISLE, the differentially expressed eccDNA-related genes were primarily involved in infectious diseases, lipid metabolism, ribosomal bioprocesses, and pathways related to neurodegenerative diseases, notably Parkinson's disease. This finding may reflect the different stages of SLE and the variations in medication composition and usage (Supplementary Figure S3) [31]. When comparing ASLE with HC, the differentially expressed genes were predominantly enriched in nucleotide metabolism, pathogenic E. coli infection, the insulin signaling pathway, and the steroid hormone pathway (Supplementary Figure S4). In the ISLE versus HC comparison, the differentially expressed genes were associated with lipid metabolism, cancer, immune molecules, and immune pathways (Supplementary Figure S5).

To exclude interference from introns and intergenic regions, we specifically screened for eccDNAs related exon region genes and identified their key biological functions and involved biological pathways using the 'clusterProfiler' package. In analyzing the differences in eccDNAs related exon region genes between ASLE and HC, we focused on 67 candidate exon-derived eccGenes with down-regulated expression and 92 genes with up-regulated expression (Figure 3a,b), Among them, exon-derived eccGenes such as TNFSF14, PLAAT4, GPER1, TRIM21, SLAMF7, and others have been proven to play a key role in the development of systemic lupus erythematosus [32–36]. GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses provided insights into the functions of these eccGenes and the biological pathways they are involved in. GO analysis revealed that these differentially expressed eccGenes were mainly localized in the outer membrane of cells, outer membrane of organelles, and circulating endosomes. Regarding Molecular Function (MF), these eccGenes were significantly involved in

type and amount of eccDNA molecules in the sample. (c) GC content per million mapped reads; (d) GC content of eccDNA in ASLE in the range of 0.01 kb to 1000 kb, ISLE and HC; (e-g) correlation analysis of GC count with the amount of C3,C4 and anti-dsDNA. (H) Venn diagrams showing the frequency of distribution of eccDNA detected in ASLE, ISLE, and HC; (i) read ratios of ASLE, ISLE, and hc-derived eccDNA mapped in specific repetitive genomic elements.

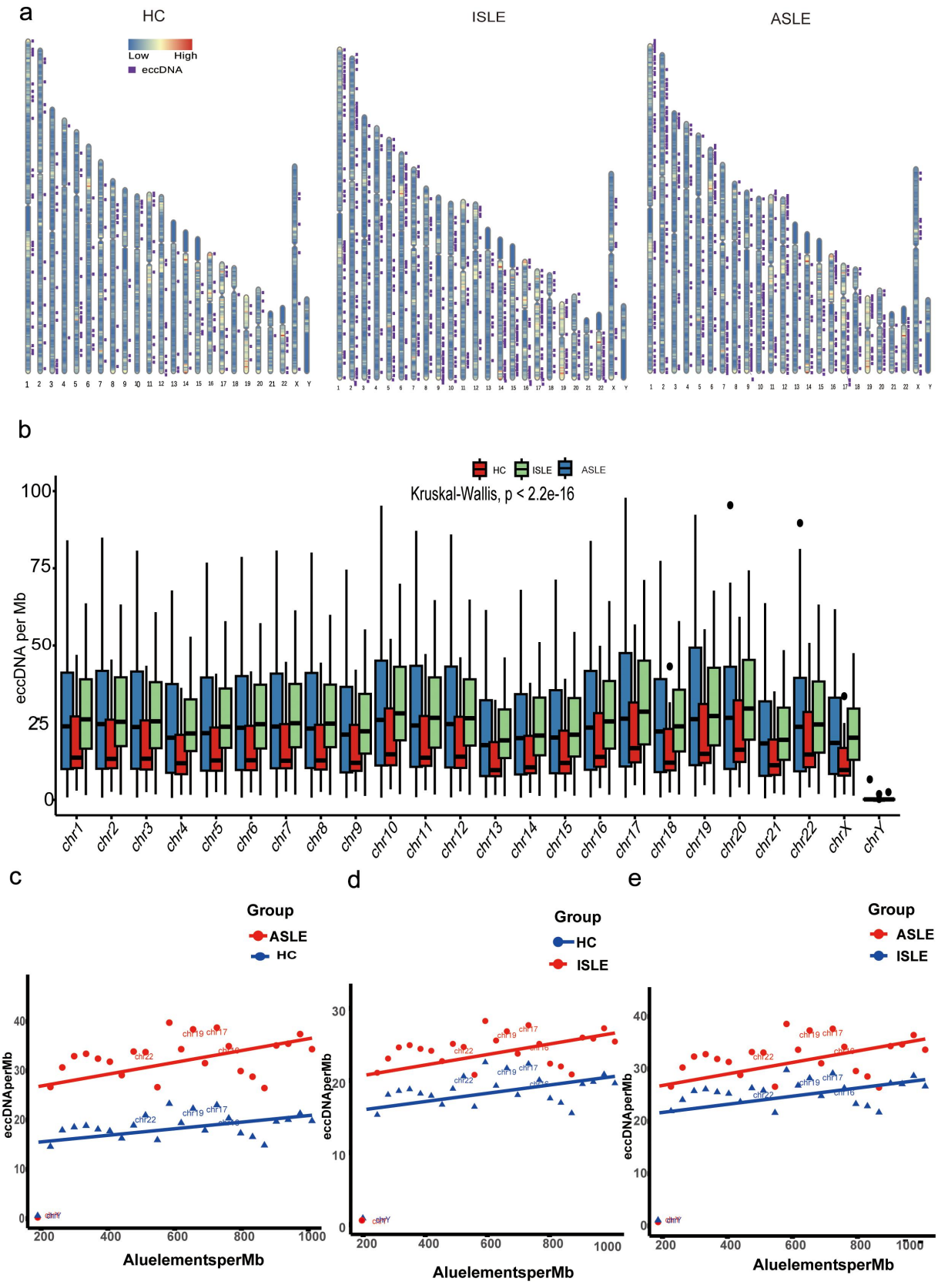

Figure 2. Frequency of eccDNA genomic distribution and its correlation with repetitive originals in ASLE and ISLE: (a) distribution of eccDNA on the genomes of ASLE, ISLE, and HC chromosomes; (b) comparison of the distribution of eccDNA on the genomes of ASLE, ISLE, and HC chromosomes, with a focus on the number of eccDNAs per megabase (mb) for each chromosome (c-d) the number of eccDNAs per mb is positively correlated with the number of SINE elements per mb.) the number of eccDNA per mb is positively correlated with the number of SINE elements per M.

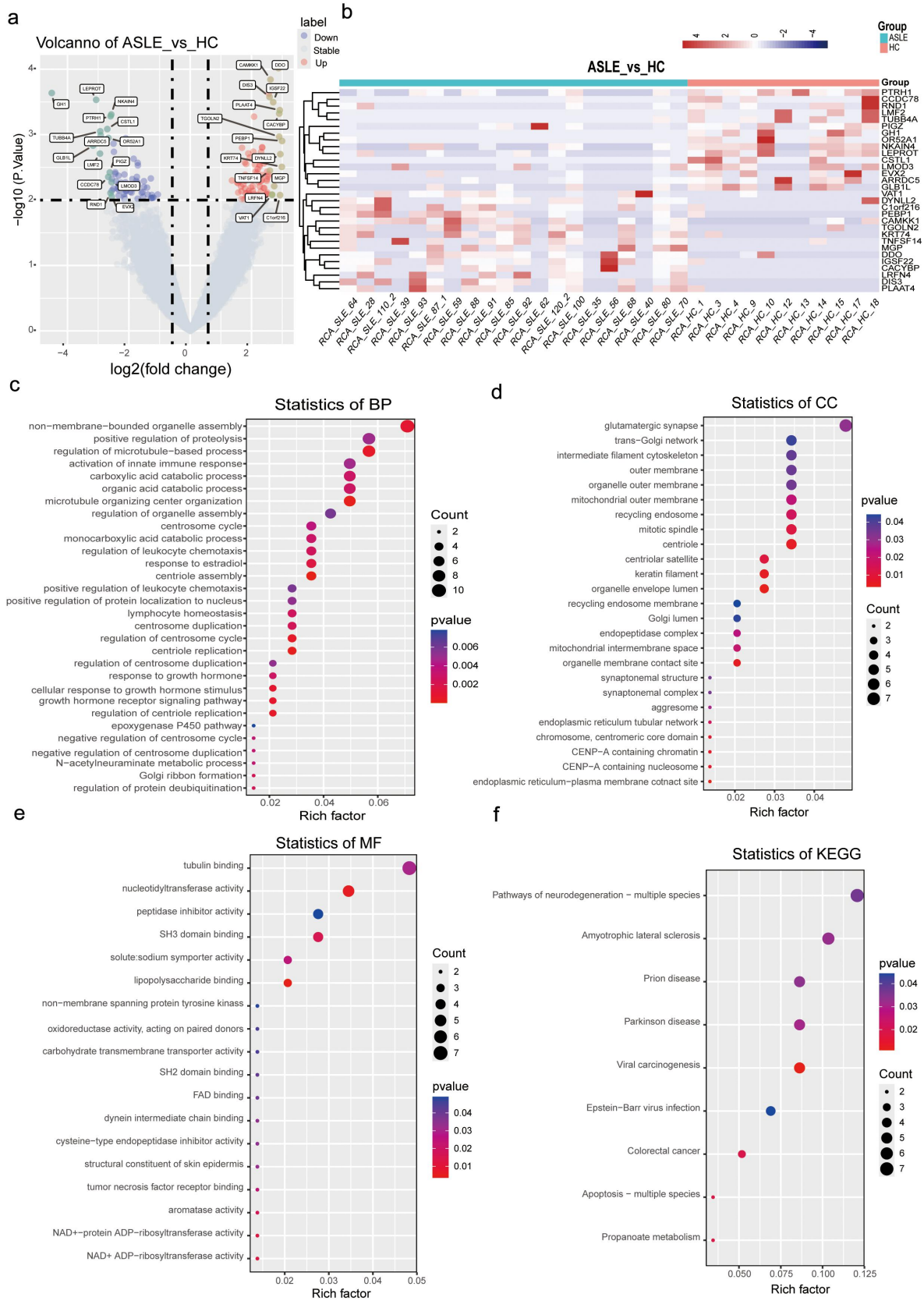

Figure 3. Differential abundance of exon-derived eccGenes in ASLE:(a) Up- and down-regulation of exon-derived eccGenes. Volcano plots show differential eccGenes. (b) Heatmap showing potential top 30 DEGs in ASLE and HC.(c-e) GO classification of eccDNAs related exon region genes specifically expressed in biological processes (c), Cellular components (d) and molecular functions (e) in ASLE patients. (f) Specific eccDNAs related exon region genes of ASLE patients are functionally enriched in KEGG.

nucleotidyltransferase activity and peptidase inhibitor activity. In terms of Biological Process (BP), these eccGenes were primarily enriched in the positive regulation of proteolysis, activation of innate immune response, and regulation of leukocyte chemotaxis (Figure 3c-e). KEGG pathway analysis further indicated that these differentially expressed eccGenes were enriched in pathways of neurodegenerative diseases, viral infections, propanoate metabolism, and apoptosis (Figure 3f).

Exon origin of eccGenes abundance differences and their biological significance between ISLE and HC

In a comparative analysis of ISLE versus HC, we identified 229 differentially expressed eccDNAs related exon region genes, with 113 down-regulated and 116 up-regulated (Figure 4a,b), In addition, eccGenes such as IRF1, IL20, MTNR1A, PROC, PPT1 and GDF5, and others, have been proven to play a critical role in the development of systemic lupus erythematosus [37–42]. GO and KEGG analyses were performed to explore the functions of these eccGenes and the associated biological pathways. Regarding CC, the differentially expressed eccGenes were mainly clustered in the nuclear membrane, mitochondrial matrix, and transport vesicles. In terms of BP, the primary functions include the negative regulation of the MAPK cascade, regulation of double-strand break repair, and the insulin receptor signaling pathway. For MF, these eccGenes are significantly involved in DNA-binding transcription activator activity and growth factor activity (Figure 4c-e). KEGG pathway analysis revealed that these eccGenes are involved in several important signaling pathways associated with SLE, including the signaling pathways regulating pluripotency of stem cell, the prolactin signaling pathway, the estrogen signaling pathway, and the JAK-STAT signaling pathway [43,44](Figure 4f).

Exon origin of eccGenes abundance differences and their biological significance between ASLE and ISLE

A total of 258 differential exon-derived eccGenes, including 70 down-regulated eccGenes and 188 up-regulated eccGenes, were identified in the exon-derived eccGenes analysis of ASLE and ISLE. The heatmap shows the top 30 differential eccGenes whose abundanc distinguishes ASLE from ISLE (Figure 5a,b). GO and KEGG analyses of these candidate eccGenes revealed significant biological insights. In the BP category, the differential eccGenes were mainly enriched in processes such as apoptosis, mitochondrial translation and negative regulation of proteolysis. In the CC category, they were mainly enriched in regions such as ribosomes, ribosomal subunits, cell membrane microdomains, and mitochondrial ribosomes. In terms of MF, these eccGenes were mainly involved in DNA-binding transcriptional repressor activity, guanyl ribonucleotide binding, and rRNA binding (Figure 5c-e).KEGG pathway analysis showed that these eccGenes were mainly associated with immune-inflammatory response pathways, Signaling pathways related to signaling, and hormone-related pathways, such as the JAK-STAT signaling pathway, the B-cell receptor signaling pathway, the estrogen signaling pathway, and the ErbB signaling pathway, among others (Figure 5f). We are concerned about the widespread impact of the JAK-STAT signaling pathway in autoimmune diseases and have shown that SLE symptoms can be alleviated by inhibiting the JAK-STAT signaling pathway [44].

Protein–protein interactions network analysis of exonic region genes of eccDNA in ASLE and ISLE

Given that exonic region genes in eccDNA encode proteins that may have significant effects on cellular function, we specifically targeted these exonic region genes for PPI analysis. To gain a deeper insight into the role of differential **exon origin of eccGenes** in the pathogenesis and progression of SLE, as well as the interactions among these key eccGenes to obtain diagnostic markers with broad roles in SLE, we conducted a PPI analysis using the STRING database. The comparative analysis of exon-derived eccGenes across ASLE-ISLE, ASLE-HC, and ISLE-HC revealed that PIK3R2, HSP90AB1, and PIK3CA stand out as the most interactive with other eccGenes (Figure 6a-c). Within these three PPI network, several eccGenes with high scores, that was, the greatest gene-to-gene linkages, including MTNR1A, GMPPB, BCL11B, GAD2, GCNT3, PIK3R2, SOS1, SIRPA, LHX5, IRX6, ISL2, TBX3, PPT1, and SERPINB3, showed significant differences in the ASLE-ISLE

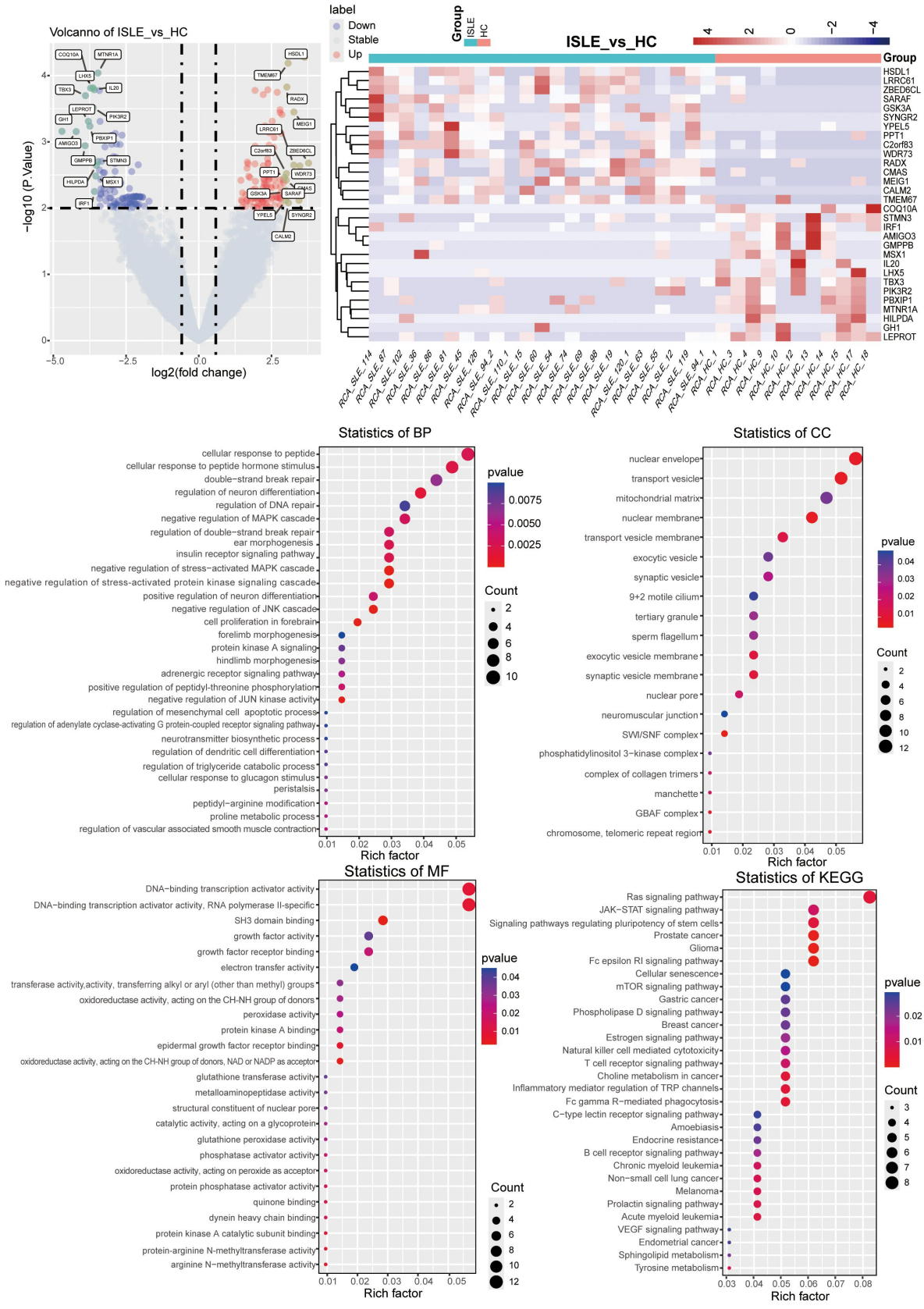

Figure 4. Exon origin of eccGenes in eccDNAs abundance differences in ISLE: (a) Up- and down-regulation of exon-derived eccGenes. Volcano plots show differential genes. (b) Heatmap showing potential top 30 exon-derived eccDNA-associated differential genes in ISLE and HC.(c-e) GO categorization of eccDNAs related exon region genes specifically expressed in biological processes (c), cellular components (d) and molecular functions (e) in ISLE patients. (f) Specific exon-derived eccGenes in ISLE patients are functionally enriched in KEGG.

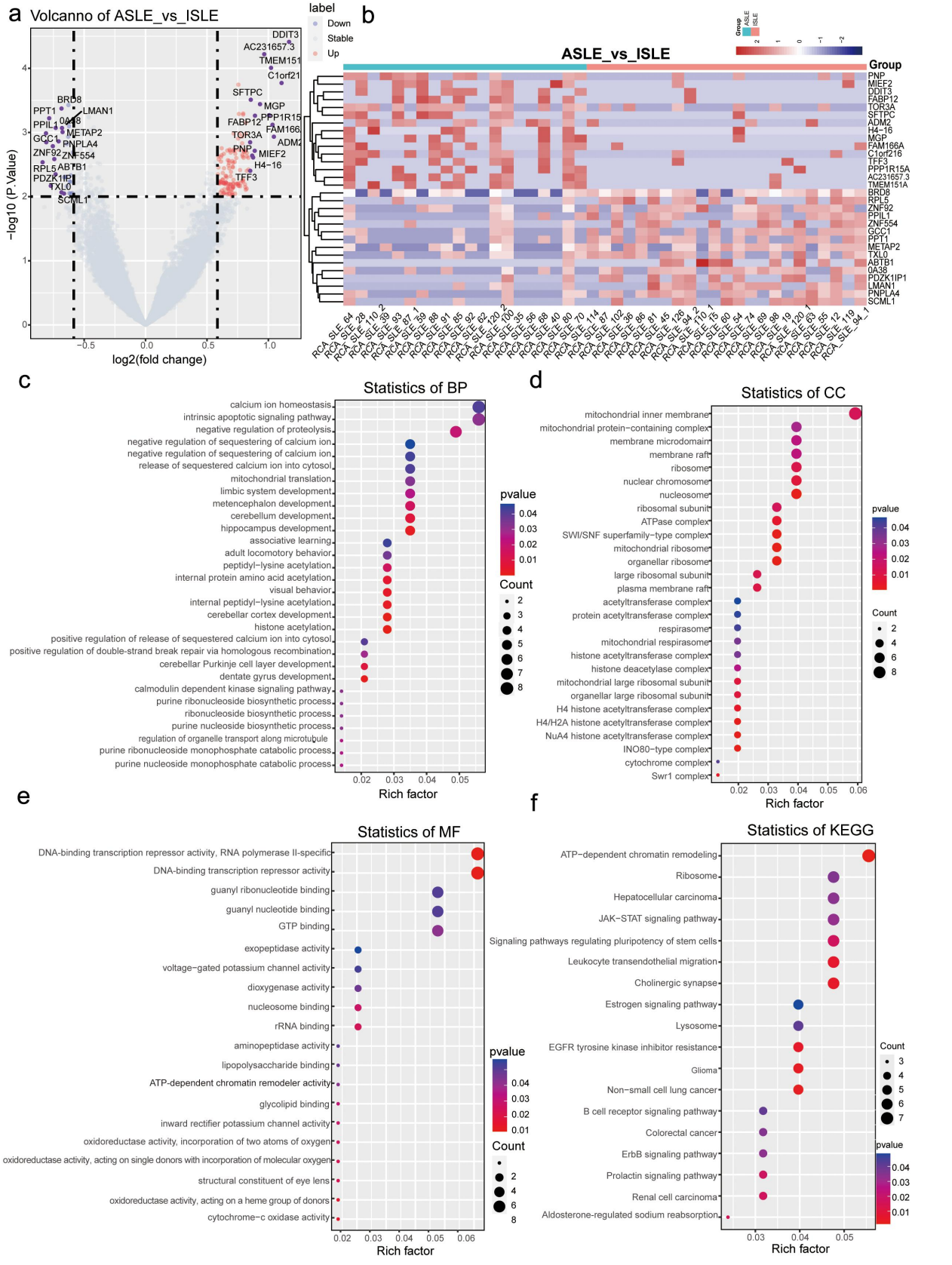

Figure 5. Exon origin of eccGenes abundance differences and their biological significance between ASLE and ISLE:(a) Up- and down-regulation of exon-derived eccGenes. Volcano plots show differential eccGenes. (b) Heatmap showing potential top 30 exon-derived eccDNA-associated differential genes in ASLE and ISLE.(c-e) GO categorization of exon-derived eccGenes specifically expressed in biological processes (c), cellular components (d), and molecular functions (e) in ASLE patients relative to ISLE patients. (f) Specific exon-derived eccGenes in KEGG functional enrichment in ASLE patients relative to ISLE patients.

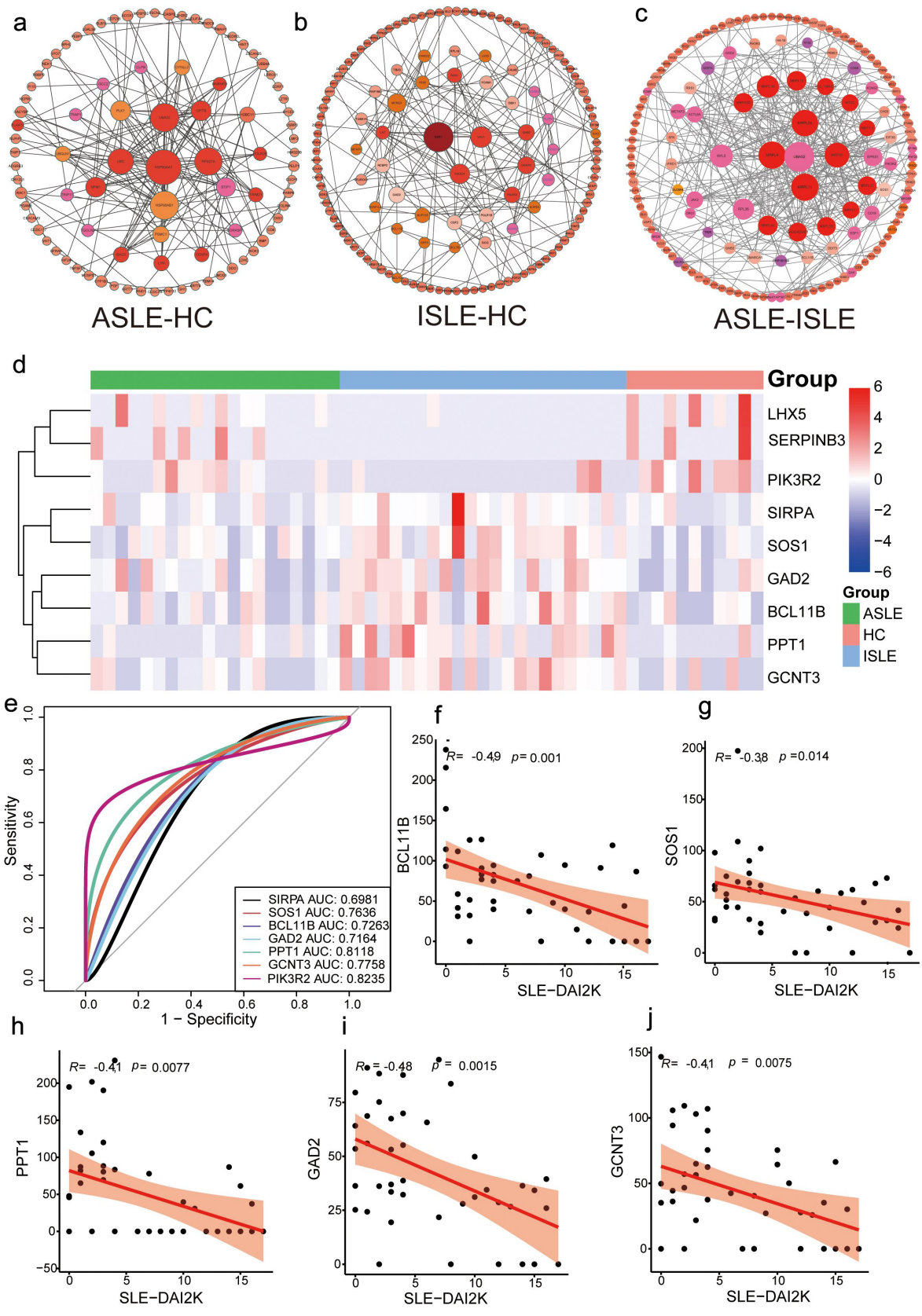

Figure 6. Protein–protein interactions network analysis of exon-derived e differential genes in ASLE and ISLE: (a-c) with differential eccDNAs related exon region genes PPI analysis of ASLE-ISLE, ASLE-HC, and ISLE-HC differential eccDNAs related exon region genes, as well as subnetworks of key gene. (d) Heatmap showing abundance of key exon-derived eccDNA-associated differential genes in ASLE, ISLE and HC groups. (e) ROC curve analysis to evaluate SIRPA, SOS1, GAD2, BCL11B, PPT1, GCNT3, and PIK3R2 as diagnostic biomarkers for differentiating ASLE from ISLE. (f-j) correlation analysis of the abundance of SOS1, GAD2, BCL11B, PPT1 and GCNT3 with SLEDAI-2K.

and ISLE-HC groups. Furthermore, based on the abundance of these eccGenes at various stages of SLE, we selected eccGenes with notable abundance variance and generated heat maps for their abundance across the three groups. The results indicated that SIRPA, SOS1, GAD2, BCL11B, PPT1, and GCNT3 were upregulated in the ISLE group compared to the ASLE and HC groups, while LHX5, SERPINB3, and PIK3R2 were downregulated in the ISLE group (Figure 6d). To further distinguish ISLE from ASLE using eccGenes, we employed the receiver operating characteristic (ROC) curve to assess the potential Exon-derived eccGenes, including SIRPA, SOS1, GAD2, BCL11B, PPT1, GCNT3, and PIK3R2, to serve as biomarkers. As shown in the figure, these genes demonstrated the potential to differentiate ISLE from ASLE, with AUC values of 0.698, 0.764, 0.726, 0.716, 0.812, 0.776, and 0.824, respectively (Figure 6e). However LHX5 and SERPINB3 do not have potential as biomarkers (Supplementary Figure S6A) Subsequently, we analyzed the correlation between clinical characteristics and these biomarkers using correlation analysis diagrams (Figures 6f-j). We found that SIRPA, SOS1, GAD2, BCL11B, GCNT3, and PPT1 were negatively correlated with SLE-DAI2K, suggesting their involvement in the occurrence of ISLE (The coordinates of the 5 exon-derived eccGenes are shown in Supplementary Table S2), whereas SIRPA and PIK3R2 did not significantly correlate with SLEDAI-2K (Supplementary Figure S6B, Supplementary Figure S6C). Among them, the abundance level of BCL11B is closely associated with the JAK-STAT signaling pathway; and SOS1 signaling promotes Stat3 activation [45,46]. Therefore, these identified eccGenes biomarkers are not only powerful tools for assessing disease activity in SLE patients, but also, through further research, they are expected to be new strategies for the treatment of SLE.

Discussion

SLE is a complex systemic autoimmune disease [47] that necessitates accurate assessment of disease activity to guide clinicians in diagnostic and therapeutic decision-making and to facilitate comparative analyses in clinical studies [48]. Real-time prediction of changes in SLE disease activity is crucial for implementing closer monitoring and prospective treatment. However, the SLEDAI-2K has deficiencies such as its inability to reflect changes in specific organ systems. Accurate clinical differentiation between quiescent and SLE is essential for developing individualized treatment plans, monitoring the condition, preventing complications, improving quality of life, evaluating prognosis, providing patient education, managing medication side effects, and offering psychological support. In this case, blood biomarkers can assist in assessing the heterogeneity and disease activity of systemic lupus erythematosus based on existing staging, potentially providing a means to predict the onset of clinical episodes several weeks in advance. Although studies have demonstrated that SLE activity with dendritic cytopenia and cfDNA can be used as biomarkers for different active phases of SLE [49,50], the cyclic structure of eccDNA and its function in autoimmune diseases make it a potential biomarker for SLE [51]. Recent studies have highlighted the significant potential of extrachromosomal circular DNA (eccDNA) in oncology, inflammatory diseases, and autoimmune diseases [52]. Studies on the differential expression and molecular mechanisms of eccDNA in disease processes have provided new insights into understanding the development of SLE [53]. The cyclic structure of eccDNA and its function in autoimmune diseases make it a potential biomarker for SLE. A total of 23 ISLE patients, 20 ASLE patients, and 11 healthy controls were recruited for this study, from which eccDNA samples were extracted and purified. To our knowledge, this is the first study to utilize eccDNA as a biomarker for the diagnosis of SLE.

Using the circle-seq technique, we analyzed the characteristics of eccDNA, including its abundance, length distribution, constituents, motifs, and functional genes in ASLE and ISLE patients as well as in healthy controls. The study findings indicate that there is no significant variation in the count values of eccDNA among ASLE, ISLE patients and three groups of healthy controls. However, the GC content distribution of eccDNA exhibits noticeable differences between the SLE and HC groups, suggesting that while the chromosomes remain stable during the disease process, there may be underlying DNA damage [54,55], In addition we found that the GC content of eccDNA has the potential to differentiate between SLE

and HC by correlation analysis of GC content with C3, C4 and Anti-dsDNA. These eccDNA differences in size, composition, and chromosomal distribution further demonstrate its potential role in the pathogenesis of SLE. Furthermore, we analyzed the role of specific eccDNA-related genes (eccGenes) in the disease process. By comparing ASLE, ISLE, and HC, we identified 158, 141, and 161 eccGenes with significantly different abundance in ASLE versus ISLE, ASLE versus HC, and ISLE versus HC, respectively. In identifying differentially abundance eccGenes among various groups, it was observed that the abundance of GH1 and LEPROT eccGenes was downregulated in ASLE compared to HC and ISLE compared to HC. This suggests that they may be involved in the pathological process of systemic lupus erythematosus.

In our functional enrichment analysis, we discovered that exon-difference eccDNA-related genes in ASLE versus ISLE and ISLE versus HC were implicated in several common biological pathways. These pathways include the JAK-STAT signaling pathway, the B-cell receptor signaling pathway, the estrogen signaling pathway, the prolactin signaling pathway, and the pathway regulating stem cell pluripotency. Disorders of these pathways and mutations in related genes are closely linked to immune activation and the progression of SLE [56–60]. By analyzing the PPI network of three groups of eccDNA (exon)-related genes and mapping the heat maps, we identified several key eccDNA genes that showed significant differences in the ASLE-ISLE and ISLE-HC groups. SIRPA, SOS1, GAD2, BCL11B, PPT1, and GCNT3 exhibited higher abundance in the ISLE group compared to the ASLE and HC groups, whereas LHX5, SERPINB3, and PIK3R2 showed significantly lower abundance in the ISLE group compared to the ASLE and HC groups. By constructing ROC curves, we evaluated the potential of SIRPA, SOS1, GAD2, BCL11B, PPT1, GCNT3 and PIK3R2 as biomarkers. The abundance levels of the biomarkers SOS1, GAD2, BCL11B, PPT1, and GCNT3 were closely associated with SLEDAI-2K, a finding that suggests their potential role in differentiating ASLE from ISLE. Studies have shown that SOS1, BCL11B, and PPT1 correlate with SLE [42,61,62], In addition, BCL11B, and SOS1 were strongly associated with the JAK-STAT signaling pathway [45,46]. This association suggests that these molecules may be involved in the molecular mechanisms of the disease by modulating the JAK-STAT signaling pathway, thus playing an important role in the pathology of SLE, which validates the reliability of our study. Our study contributes to an in-depth understanding of the molecular mechanisms of SLE and may reveal new therapeutic targets. However, there are some limitations to this study. First, we only focused on eccDNA-related genes when analyzing biomarkers and did not investigate the specific differences of eccDNAs in disease development. Second, due to research funding and time constraints, A shortcoming of our research is the absence of experimental validation using our own samples. While we relied on circle-seq results and an RNA-seq dataset to support our arguments, to more effectively validate our research findings, it is necessary to obtain clinical samples for biological validation and to thoroughly investigate the specific functions of genes associated with eccDNA.

Expanding and deepening future research in response to these limitations could be highly beneficial. Firstly, considering more eccDNA-specific analyses that focus on how eccDNA expression profiles change during disease development could provide valuable insights into the role of eccDNA in SLE progression. Secondly, exploring alternative research methods or techniques to validate the accuracy of the results and assess their applicability in different contexts could enhance the robustness of the findings. Finally, incorporating additional variables and dimensions in future studies could lead to a more comprehensive analysis and a deeper understanding of the research phenomenon.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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