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Characterization of Immune-Related circRNAs and mRNAs in Human Chronic Atrophic Gastritis

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Background: Chronic atrophic gastritis (CAG) is a severe condition characterized by inflammation and loss of appropriate mucosal glands in the stomach. The underlying mechanisms of CAG development remain unclear. Exploring immune-related circular RNAs (circRNAs) could provide insights for potential diagnostic and therapeutic strategies.

Methods: Samples from 40 patients with CAG and non-CAG (CNAG) underwent high-throughput sequencing, and EdgeR analysis identified differentially expressed circRNAs and mRNAs. Gene Ontology (GO) analysis elucidated biological functions, while Immune Cell Abundance Identifier (ImmuCellAI) estimated immune cell abundance. Flow cytometry analyzed immune cell infiltration. Weighted gene co-expression network analysis (WGCNA) identified hub genes related to the immune response in CAG. CircRNA-mRNA networks were constructed, and qRT-PCR validated findings.

Results: A total of 163 differentially expressed immune-related genes (DEIRGs) were identified between CAG and CNAG. The upregulated immune-related mRNAs in CAG were significantly enriched in antimicrobial humoral response, viral entry into host cells, neutrophil activation, and leukocyte migration. Conversely, downregulated immune-related mRNAs were linked to regulation of natural killer cell-mediated cytotoxicity, positive regulation of adaptive immune response, antigen receptor-mediated signaling pathway, and B cell activation. Immune Cell Abundance Identifier (ImmuCellAI) and flow cytometry confirmed increased neutrophil infiltration in CAG compared to CNAG. WGCNA identified 56 hub immune-related genes. Additionally, circRNA expression profiles in CNAG and CAG were explored, with 19 upregulated and 23 downregulated circRNAs identified in CAG. The upregulated circRNAs were associated with biological processes like carnitine metabolic process and regulation of B cell receptor signaling pathway. A circRNA-mRNA co-expression network was constructed based on five circRNAs highly related to hub immune-related genes. Furthermore, the expression of eight immune-related mRNAs and five circRNAs were validated in CAG.

Conclusion: This study is the first systematic analysis of circRNA profiles in CAG and provide important insights for potential immunotherapeutic strategies and early diagnostic biomarkers in CAG treatment.

Keywords: chronic atrophic gastritis, circRNAs, immune-related genes, immune cell infiltration

Introduction

CAG is the final consequence of an inflammatory process that ultimately leads to loss of appropriate mucosal glands. Numerous studies have indicated that chronic atrophic gastritis (CAG) can be attributed to various factors, including immune-mediated inflammation, mucosal gland atrophy, increase of serum autoantibodies to gastric parietal cells and vitamin B12 deficiency. The process of CNAG has been proposed to occur through three stages: glandular atrophy, metaplasia dysplasia, and, ultimately, gastric cancer (GC).[1,2] CAG has a global prevalence of around 20–30%, with significantly higher rates in regions such as East Asia. In China, the prevalence of CAG in adults has been reported to be as high as 32.5%. Furthermore, studies have shown that patients with CAG have a higher risk of progressing to GC, especially if Helicobacter pylori infection is involved. According to reports from Global Cancer Statistics 2020,[3] GC is the fourth leading cause of cancer death among all cancer types. As the precancerous lesion of GC, early diagnosis and treatment of CAG can play a crucial role in the prevention of gastric carcinogenesis. Despite the recognized association

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between CAG and gastric carcinogenesis, the precise pathogenetic mechanisms governing CAG development remain poorly understood, thus impeding the development of effective therapeutic strategies for its management. CAG is more serious than CNAG, its pathogenesis may associate with various immune cells and genes. CAG is characterized by chronic inflammation and infiltration of immune cells, such as central memory CD4 T cells and monocytes, in the gastric mucosa. In contrast, CNAG typically exhibits a milder and less pronounced immune response.[4]

Chemokines and interleukins were inextricably linked to the development of immunoinflammatory cells and diseases. In the transition from CAG to early and midstage gastric cancer, distinct changes in the expression patterns of these cytokines were observed, showing a "reverse expression" phenomenon.[4] Interferon-gamma (IFN-γ), a type 1 cytokine known for its involvement in autoimmunity and infection, has been studied for its direct impact on gastric epithelial cells. It has been identified as a significant contributor in promoting parietal cell atrophy and metaplasia during the progression from gastritis to gastric atrophy and metaplasia.[5]

Circular RNAs (circRNAs) are a class of non-coding RNAs first discovered by Sanger et al in 1976. CircRNA was once considered as viral genomes or byproducts of mis-splicing events with no biological function. CircRNAs can be classified into three types based on their composition: exon circRNA (ecRNA), intron circRNA (ciRNA), and exon-intron circRNA (ElciRNA). CircRNA have no 5′ cap or 3′ poly(A) tail, it is a closed ring structure formed by cyclic covalent bonds, therefore the structure can resist exonucleolytic degradation with high stability. Indeed, with the advancements in high-throughput sequencing and bioinformatics analysis, the understanding of circRNAs has expanded significantly. Increasing evidence suggests that circRNAs are not just by-products or "splicing noise" but rather an important component of the noncoding RNA family with significant functional potential. Numerous studies have demonstrated the relevance of circRNAs in the occurrence and progression of various human diseases, including cancer,[6–14] nervous system diseases,[15–18] cardiovascular and cerebrovascular diseases,[19–22] among others.

One specific circRNA, hsa_circ_0014717, has been identified as stably present in human gastric juice and shows a significant decrease in patients with CAG.[23] Although studies have identified specific circRNAs that are differentially expressed in CAG and have shown their potential as biomarkers, the exact mechanisms by which circRNAs contribute to CAG pathogenesis and immune response regulation are not yet fully understood. Here, we characterize the expression of circRNAs and identify specific circRNAs that are associated with immune-related processes in CAG.

Materials and Methods

Sample Collection

A total of 40 patients were included in this study, including 20 patients with CNAG and 20 patients with CAG. All patients were from the China-Japan Union Hospital of Jilin University. Informed consent for inclusion was provided to all subjects prior to their participation in the study. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Jilin University (approval number: 2019082110). The detailed procedures for specimen collection were same as the procedures of previous studies.[24]

Identification of Differentially Expressed circRNAs and mRNAs

We conducted high-throughput sequencing and submitted the raw data to the Gene Expression Omnibus (GEO) database (accession numbers GSE153224)[24] and Genome Sequence Archive (GSA) for Human database (accession numbers HRA005806). We utilized EdgeR software (v3.16.5) to normalize the data from these publicly available datasets and to perform the analysis of differentially expressed circRNAs. The significance of the changes in the expression profile of the transcripts between groups was determined by a statistical test, by which the differentially expressed circRNAs were identified. The HeatMap2 function in the R package was used for cluster analysis of differentially expressed circRNAs with FPKM values. When the two groups of samples are compared, the volcano map is drawn in R through multiple changes and p-value. To display the distribution of circRNA and mRNAs along the chromosomes more intuitively, we used Circos software (version: circos-0.69–6) to map the genomic locations of circRNAs and mRNAs screened as described above. The threshold set for the significantly differentially expressed circRNAs and mRNAs was a fold change of ≥ 2.0 and a p-value of < 0.05.

Functional Enrichment Analysis

Gene ontology (http://www.geneontology.org) is an online analytical tool used to extract comprehensive biological information associated with large candidate gene lists. GO analysis of the target genes of the differentially expressed circRNAs, immune related mRNA and 12 modules screened by WGCNA was performed in this study. By bioinformatics analysis, GO terms were selected from the significantly enriched gene sets ($P<0.05$).

Immune Cell Infiltration of CAG and CNAG

Immune Cell Abundance Identifier (ImmuCellAI http://bioinfo.life.hust.edu.cn/web/ ImmuCellAI/)[25] is a tool to estimate the abundance of 24 immune cells from gene expression dataset including RNA-Seq and microarray data, in which the 24 immune cells are comprised of 18 T-cell subtypes and 6 other immune cells: B cell, NK cell, Monocyte cell, Macrophage cell, Neutrophil cell and DC cell.

Evaluation of Th, Tc, Neutrophils and NK Cell Lineages by Flow Cytometry

All flow cytometry assays were performed using BD biosciences' clone UCHT1 (CD66b-FITC, CD16-PE, CD3-Percp–cy5.5, CD4-PE, CD8-FITC, CD56-FITC). All reagents and detailed instructions are provided in the user guides for each kit. Please refer to each clone UCHT1's specific instruction for more detailed information. All gastric tissue samples acquisition and data analysis were performed using the Guava easyCyte-Luminex China system. At last, we used software FlowJo (version 10.8.0) to analyze flow cytometry data.

Weighted Gene Co-Expression Network Analysis (WGCNA)

WGCNA is a popular systems biology method used to not only construct gene networks but also detect gene modules and identify the central players within modules. We used edger and R to get 1145 mRNAs list that were up regulated and down, and WGCNA algorithm was run with these mRNAs. We used the "WGCNA" package in R to explore the key genes and modules related to clinical traits. We assumed signed correlation networks and computed bi-weight mid-correlations between CAG tissue genes. A threshold of 0.9 was used for the scale free topology index to set the soft thresholding power, which was set to 5. Recommendations from the tutorial were used for settings related to dendrogram cutting and module merging. Module eigengenes (first principal component of genes included in the module) were then correlated with each phenotype using Spearman correlation. The function of each module was characterized using gene ontology (GO) enrichment.

Construction of the circRNA–mRNA Network

Co-expression analysis was implemented by calculating the Pearson correlation coefficient (PCC) based on the expression levels of circRNAs and mRNAs. A statistically significant correlation pair was indicated by $PCC > 0.99$ or $P < -0.99$ and $P<0.01$.

qRT-PCR

The SYBR green qRT-PCR assay was used to confirm the differentially expressed circRNAs and mRNAs identified by high-throughput whole-transcriptome sequencing. Tissue samples were collected from 20 CAG and 20 CNAG patients. The total RNA isolated from the tissue sample of an individual patient was used for reverse transcription and PCR. qRT-PCR was performed using the SYBR green assay kit (TaKaRa Biotechnology, Dalian, China) and a Roche LightCycler 480 instrument (Roche Applied Science, Mannheim, Germany). The five specific primers of circRNAs and mRNAs used for qRT-PCR are listed in Supplementary Table S1. The qRT-PCR program was as follows: denaturation at 95°C for 30s, followed by 40 cycles at 95°C for 5s and 60 °C for 30s. Each sample was run in triplicate. The relative amount of lncRNA was normalized against that of GAPDH, and the fold change for each lncRNA was calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data were expressed as the mean ± standard deviation. The fold change value and P-value were used to evaluate the differentially expressed circRNAs and mRNAs. The significance of the difference of the expression levels of circRNAs

and mRNAs between the CAG and CNAG groups was estimated using the Student's *t*-test. The false discovery rate (FDR) was calculated to correct the P-value. P<0.05 was regarded as statistically significant.

Results

Functional Analysis of Immune-Related Differentially Expressed mRNAs

As shown in Figure 1A, the diagnosis of CNAG or CAG was confirmed based on the endoscopic and pathological examination. RNA-sequencing was performed on samples from 20 patients with CAG and 20 patients with CNAG. The differentially expressed genes between CAG and CNAG were analyzed (Figure 1B), and immune related genes were downloaded from IMMPORT database. Then, these differentially expressed genes were intersected with immune related genes. A total of 163 differential expressed immune-related genes (DEIRGs) were screened, which included 76 upregulated and 87 downregulated genes (Figure 1C). The chosen DEIRGs were then functionally analyzed using the GO databases. The result showed that the upregulated immune-related mRNAs in CAG were significantly enriched in the following four biological processes: antimicrobial humoral response, viral entry into host cell, neutrophil activation, and leukocyte migration (Figure 1D). Meanwhile, the top four biological processes related to the downregulated immune-related mRNAs in CAG were regulation of natural killer cell mediated cytotoxicity, positive regulation of adaptive immune response, antigen receptor-mediated signaling pathway, and B cell activation (Figure 1E).

Infiltrating Immune Cells in CAG and CNAG

As previously described, we found that the DEIRGs of CAG were involved in neutrophil and lymphocyte functions. Thus, we assessed the immune cell infiltration characteristics in CAG. Immune Cell Abundance Identifier (ImmuCellAI)

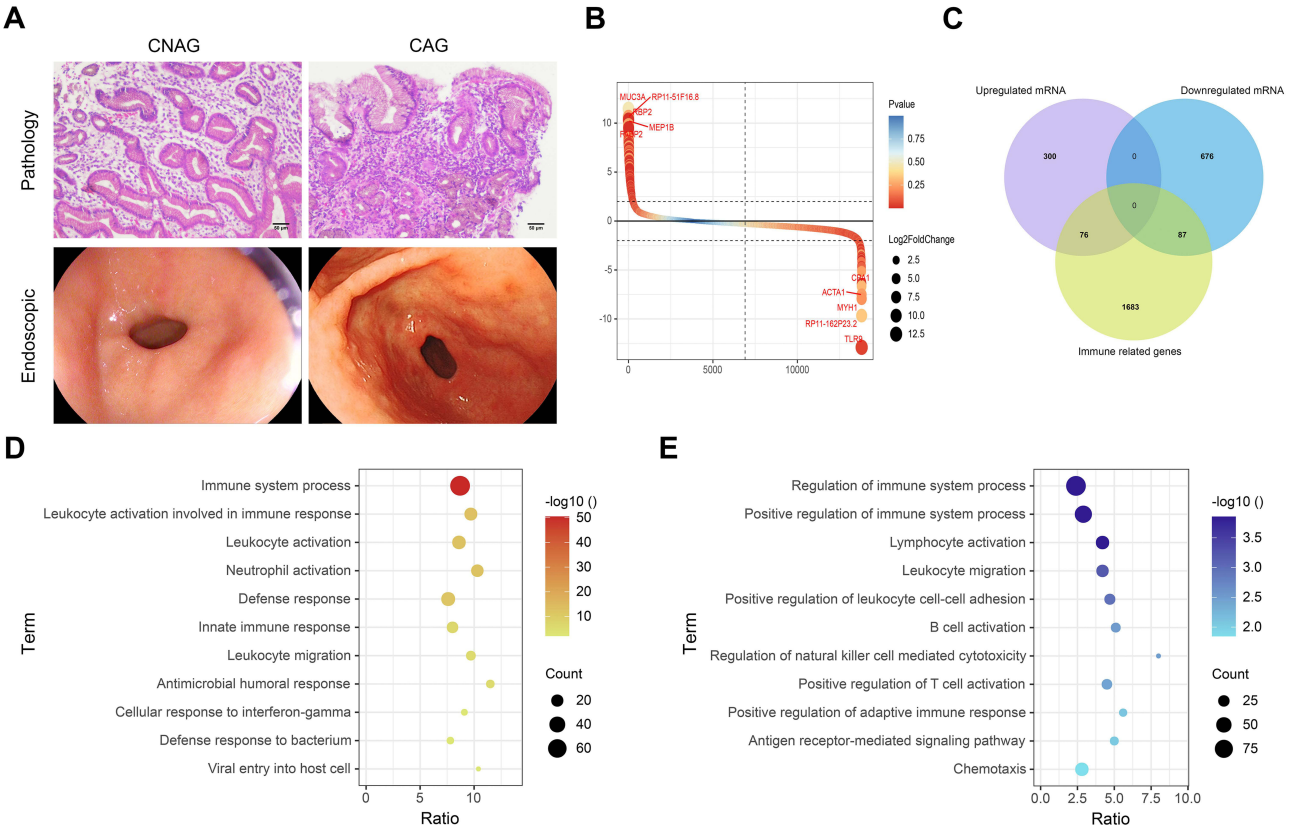

Figure 1 (**A**) Pathological and endoscopic examination of CAG or CNAG. H&E staining showing gastric mucosa. In CAG, there is marked infiltration of immune cells in the lamina propria, with a reduction in the number of gastric glands. Scale bar = 50 μm. Endoscopic images of gastric mucosa in CNAG and CAG. (**B**) Volcano plot showing differentially expressed genes in CAG; circle size reflects -log10(*P*-value), with red for upregulated and blue for downregulated genes. (**C**) Venn diagram illustrating the overlap of upregulated mRNAs, downregulated mRNAs, and immune-related mRNAs in CAG. (**D**) Top 10 GO terms for upregulated immune-related mRNAs in CAG; circle size indicates gene count, and color represents -log10(P-value). (**E**) Top 10 GO terms for downregulated immune-related mRNAs; size shows gene count, color shows significance.

was used to analyze the relative abundance of 24 human immune cell types. As shown in Figure 2A, the infiltration of Tcm, NKT, CD8 T cells were decreased in CAG tissues than in CNAG, while the infiltration of neutrophils was increased. These results were further validated by flow cytometry. The result showed that patients with CAG displayed higher neutrophil percentage than patients with CNAG, while there was no significantly difference in NK cells, Th cells and Tc cell (Figure 2B).

Identification of Key Genes in CAG by WGCNA Analysis

Weighted Gene Co-expression Network Analysis (WGCNA) was performed for the identification of crucial gene modules and key genes. To merge the highly familiar modules, we chose a cut line of <0.25 and a minimum module size of 30 using the dynamic hybrid tree cut method. 9 gene co-expression modules were finally constructed, and different colors represent different modules (Figure 3A and B). Next, the interested modules from GSE153224 dataset were found to have the highly correlation (including positive and negative correlation) with CAG. We conducted a GO analysis of genes in the modules black, green, and yellow to gain insights into their functional implications (Figure 3C). Then, 56 hub immune-related genes were identified by WGCNA (Figure 3D). These hub genes may be key players in the immune response associated with CAG.

Comparison of circRNAs

We analyzed the expression characteristics of these circRNAs regarding their distribution in terms of length. A total of 6,245 circRNAs were obtained from all samples which can be downloaded from GSA database. All circRNAs ranged in length

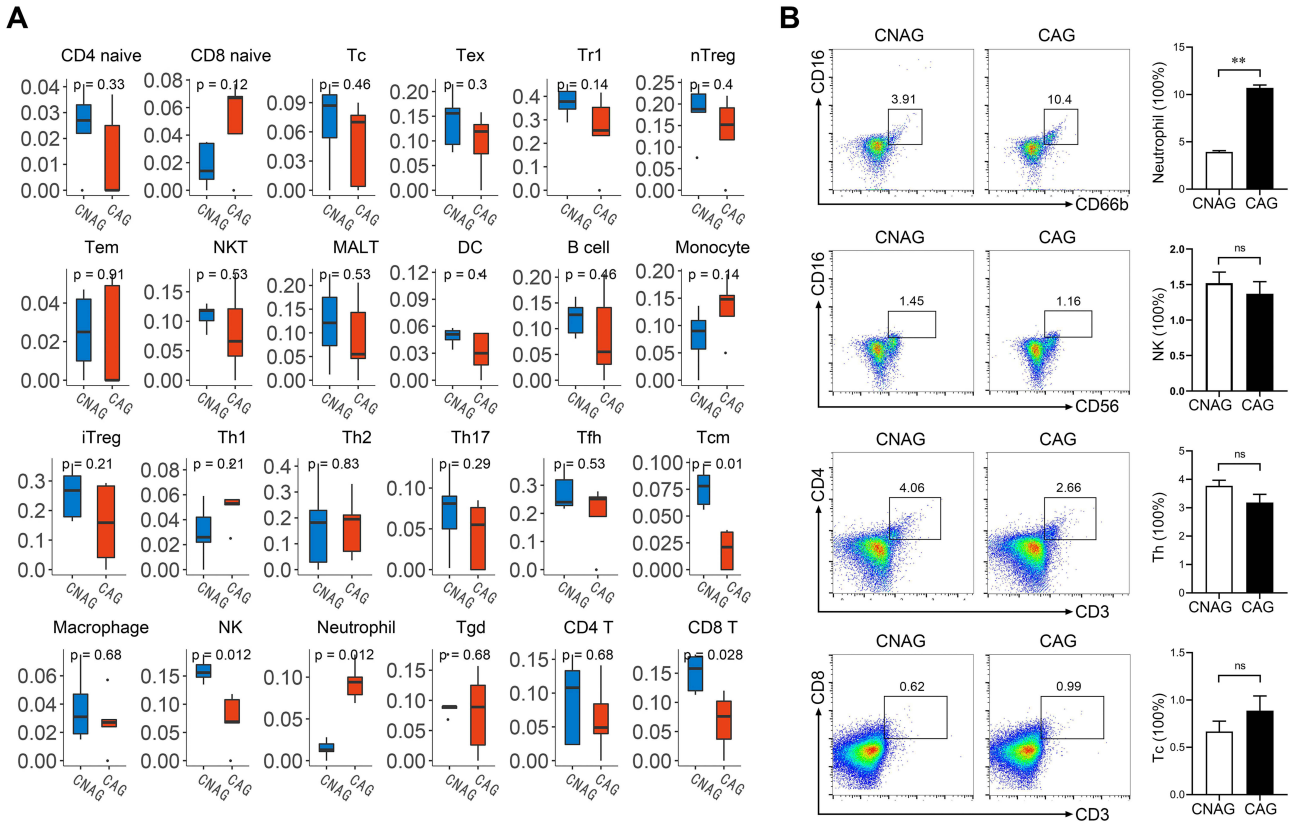

Figure 2 (**A**) Evaluation results of immune cell proportions in CAG and CNAG samples using ImmuCellAI. (**B**) Flow cytometry analysis of neutrophils, NK cells, Th cells, and Tc cells in CAG and CNAG. Statistical significance was assessed using the two-tailed *t*-test, with P < 0.05 indicating significance. A significant increase in neutrophil proportion was observed in the CAG group (**P<0.01).

Abbreviations: Tc, cytotoxic T cell; Tex, exhausted T cell; Tr1, type 1 regulatory T cell; nTreg, natural regulatory T cell; Tem, effector memory T cell; NKT, natural killer T cell; MAIT, mucosal-associated invariant T cell; DC, dendritic cell; iTreg, induced regulatory T cell; Th, helper T cell; Tfh, T follicular helper cell; Tcm, central memory T cell; Tgd, gamma-delta T cell.

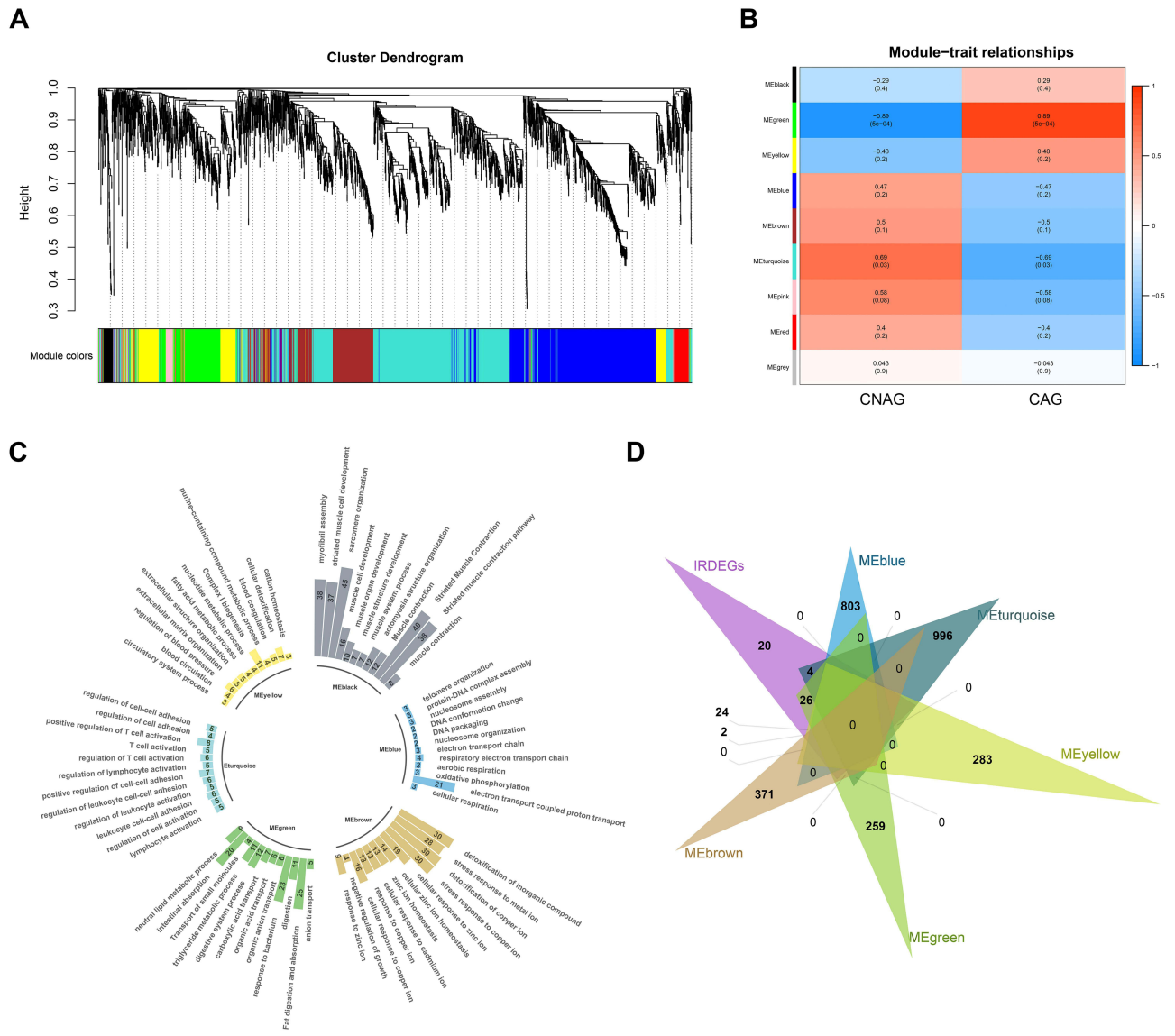

Figure 3 (**A**) Clustering of differentially expressed mRNAs and module screening based on gene expression patterns. The top panel represents the gene dendrogram, while the bottom panel displays the identified gene modules, each denoted by a unique color. A total of 9 distinct modules were identified. (**B**) Module-trait relationships. Rows represent color-coded modules, and columns represent clinical traits (CAG and CNAG). (**C**) GO enrichment analysis of each module. (**D**) Venn diagram showing the overlap between DEIRGs and key genes identified through WGCNA, with numbers representing gene counts in each category.

from 80 to more than 6,000 nt, and most of the circRNA were 200–3,000 nt long (Figure 4A). All mRNAs ranged in length from 80 to more than 6,000 nt, and most of the mRNAs were more than 6,000 nt (Figure 4B). These were collected from the authoritative database circBase as well as some data reported in the literature (Figure 4C). According to the relative chromosomal position of the coding gene, circRNAs can be classified into six broad categories: exonic, sense-overlapping, intronic, antisense, and intergenic. The 6,245 circRNAs included 3,886 exonic circRNAs (62.2%), 1,156 sense-overlapping (18.5%), 1,091 intronic circRNAs (17.5%), 57 antisense circRNAs (0.9%), and 55 intergenic circRNAs (0.9%; Figure 4D).

Identification and Functional Analysis of Differentially Expressed circRNAs in CAG

We also identified differentially expressed circRNAs between CAG and CNAG samples, which are shown by hierarchical clustering in Figure 5A. In total, 19 upregulated and 23 downregulated circRNAs in CAG were identified by the comparison of CAG and CNAG, using the following thresholds: fold change ≥2.0 and P<0.05 (Figure 5B). The Circos plot shows the genomic distribution of the mRNAs and circRNAs clusters (Figure 5C). Furthermore, the top 10

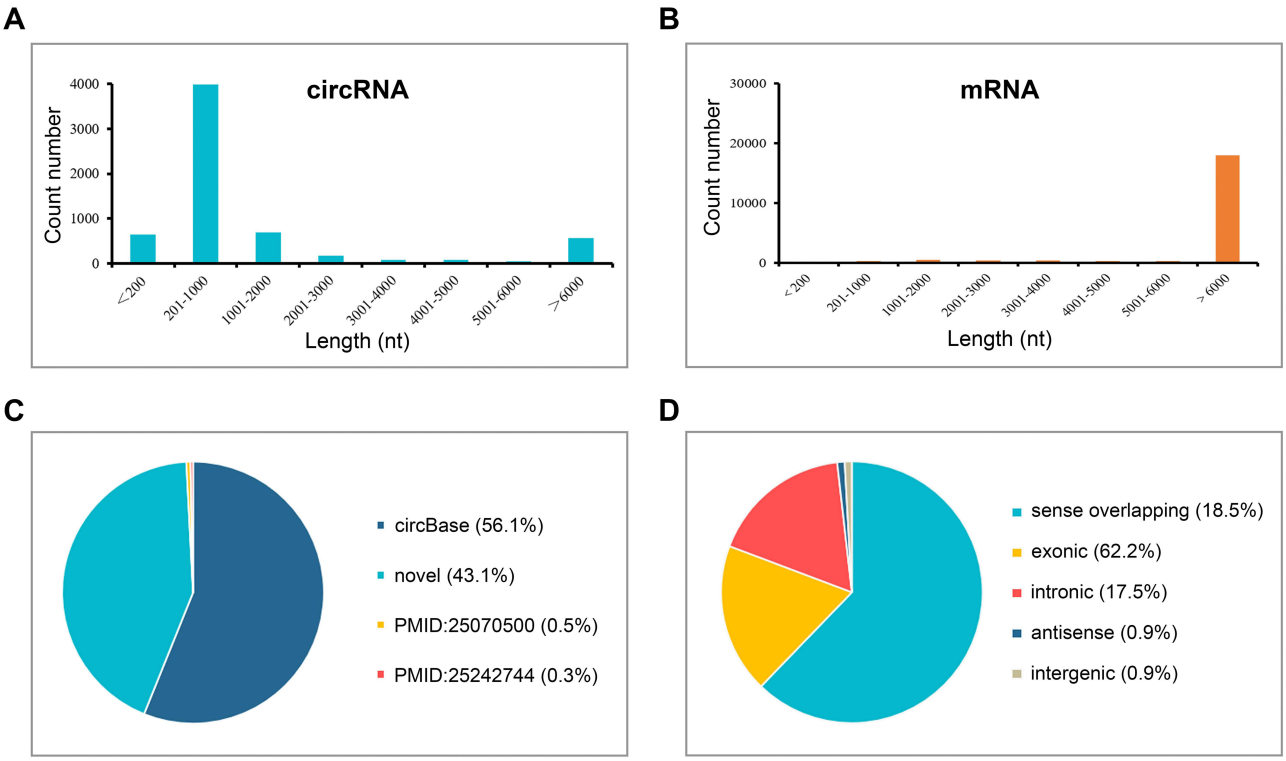

Figure 4 Characteristics of circRNAs identified in this study. (**A**) Distribution of the transcript lengths of the circRNAs. (**B**) Distribution of the transcript lengths of the mRNAs. (**C**) Pie chart showing the comparative numbers of circRNAs from authoritative databases. (**D**) Pie chart showing the components of circRNAs in each category according to their relative chromosomal position to coding genes.

upregulated and 10 downregulated circRNAs in CAG are listed in Table 1. To further illustrate the functions of the differentially expressed circRNAs in CAG, we analyzed the GO categories using a tool called Gene ontology, by which the most significant GO terms of differentially expressed circRNAs can be found. GO analysis showed that the upregulated circRNAs in CAG were significantly enriched in the following four biological processes: carnitine metabolic process, regulation of B cell receptor signaling pathway, regulation of inhibitory postsynaptic membrane potential, and smoothened signaling pathway involved in dorsal/ventral neural tube patterning (Figure 5D). Meanwhile, the top four biological processes related to the downregulated circRNAs in CAG were negative regulation of intrinsic apoptotic signaling pathway, intrinsic apoptotic signaling pathway, regulation of intrinsic apoptotic signaling pathway, and negative regulation of sequence-specific DNA binding transcription factor activity (Figure 5E).

Co-Expression Analysis of Differentially Expressed Immune-Related circRNAs and mRNAs in CAG

The mRNA-circRNA correlation was analyzed by Pearson's correlation algorithm and 16 mRNA-circRNA pairs were screened (Figure 6A). We selected 5 circRNAs from 16 that were highly related to hub immune-related mRNAs to construct a circRNA-mRNA co-expression network (Figure 6B). Next, the selected immune-related mRNAs and circRNAs were validated using quantitative PCR. As shown in Figure 6C, AGPAT2, SLC7A9, OLFM4, EPCAM, ANXA2, PRSS3, MUC12 and CD55 were upregulated in CAG, compared with CNAG. Meanwhile, as shown in Figure 6D, chrM:13298–13449+, hsa_circ_0005320, hsa_circ_0000339, chr7:100678797–100678973+, and hsa_circ_0003664 were upregulated in CAG, compared with CNAG. Therefore, the RNA-seq results are consistent with our findings using qRT-PCR.

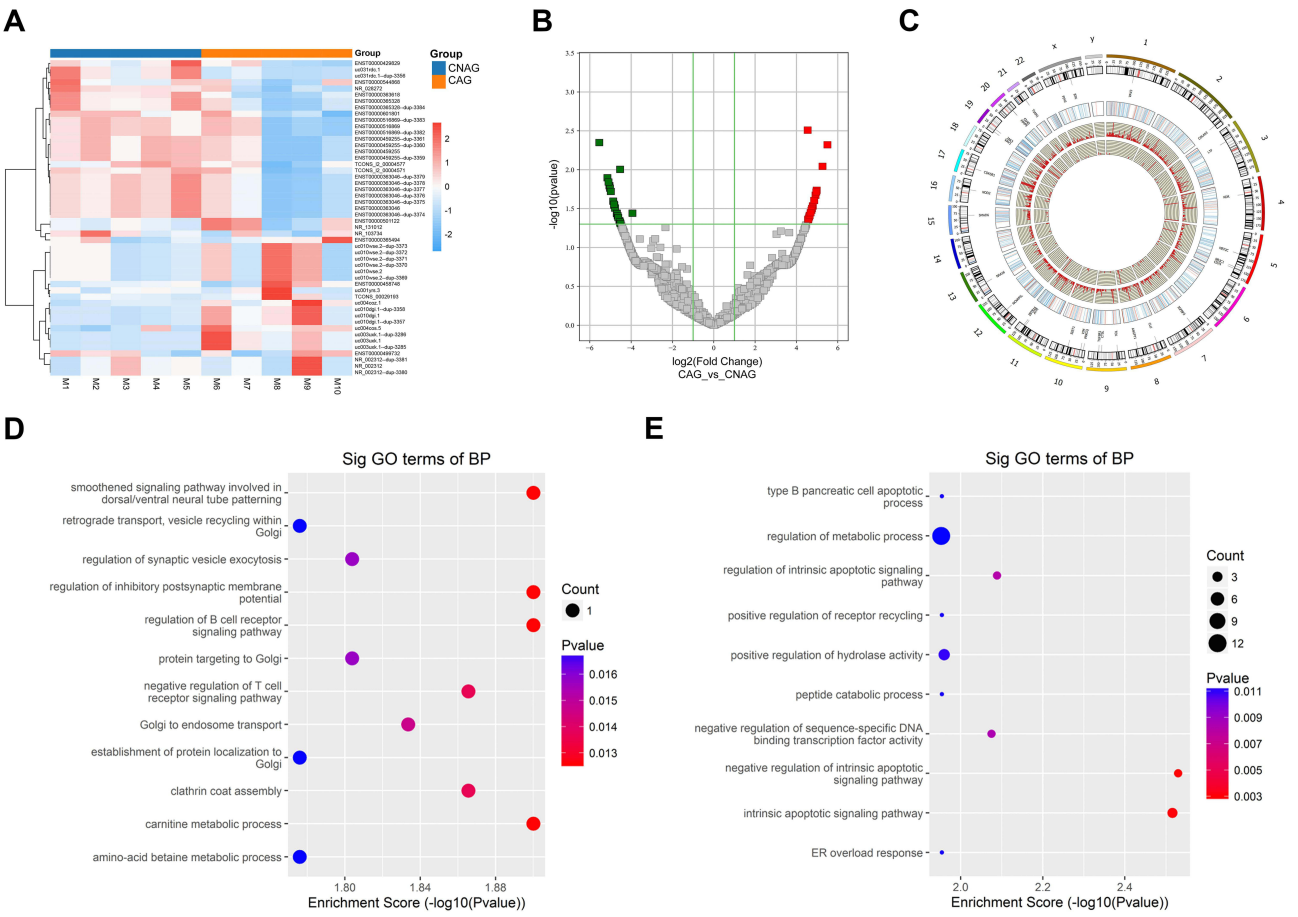

Figure 5 (**A**) Hierarchical clustering shows distinguishable expression profiles of circRNAs in the CAG and CNAG tissue samples. Upregulated expression is indicated in red, and downregulated expression is indicated in blue. (**B**) Volcano plot displaying differentially expressed circRNAs between CAG and CNAG. Red dots represent significantly upregulated circRNAs ($P < 0.05$), and green dots represent significantly downregulated circRNAs ($P < 0.05$). (**C**) Circos plot showing the mRNAs and circRNAs on human chromosomes. From the outside in, the first layer of the Circos plot is a chromosome map of the human genome. The second layer shows the up-regulated genes in red and down-regulated genes in blue in the CAG. The third and fourth layers show the fold change of all different expressed mRNAs and circRNAs. (**D**) Top 12 GO terms for upregulated circRNAs in CAG. (**E**) The top 10 GO terms of the downregulated circRNAs in CAG. Circle size reflects gene count, and color scale shows -log10 (P-value).

Discussion

GC is a malignant tumor which is the fifth most commonly diagnosed cancer and the fourth cause of death.[3] Notably, GC has the second highest incidence and third highest mortality rate of all malignancies in China.[26] Recent studies have revealed that CAG is a precancerous state of GC. In addition, it has been reported that increased severity of atrophy and extent of intestinal metaplasia is associated with an increased risk of GC.[27] Therefore, the early detection, early diagnosis, and timely treatment of such precancerous lesions may help reduce the incidence of GC.[28] Moreover, the diagnosis of CAG depends on histopathological guidance after random endoscopic biopsy sampling currently (eg, Sydney biopsy strategy). However, there are some disadvantages to this approach, such as inadequate diagnosis, lack of repeatability, and low correlation between endoscopic findings and the histological diagnosis. Thus, it is necessary to discover novel biomarkers with high sensitivity and specificity for the development of new approaches for the early diagnosis of CAG.

CircRNAs and mRNAs have been shown to play essential roles in many physiological and pathological processes.[29–34] In recent years, the importance of circRNAs in the occurrence and development of different cancers has received increasing attention. Studies have found that circRNAs actively participate in the occurrence, development and metastasis of many different tumors,[35–40] these studies indicate that circRNAs have great potential for use in human tumor prediction, and prognosis and clinical treatment.[41,42] Several studies have focused on exploring the relationship between circRNAs and GC. CircCCDC66

Table 1 The Top 10 Upregulated and 10 Downregulated circRNAs in CAG

CircRNA ID	*P*-value	logFC	CircRNA Source	CircRNA_Type
hsa_circ_0000006	0.0048	5.50	circBase	sense overlapping
hsa_circ_0005320	0.0091	5.26	circBase	exonic
hsa_circ_0003947	0.0184	4.98	circBase	exonic
hsa_circ_0001410	0.0193	4.96	circBase	exonic
hsa_circ_0003664	0.0213	4.92	circBase	exonic
hsa_circ_0003152	0.0249	4.86	circBase	exonic
hsa_circ_0006355	0.0259	4.86	circBase	sense overlapping
hsa_circ_0045096	0.0292	4.79	circBase	exonic
hsa_circ_0008518	0.0315	4.75	circBase	exonic
chr1:51868107–51,887,530-	0.0332	4.73	novel	exonic
hsa_circ_0004368	0.0045	−5.54	circBase	exonic
hsa_circ_0003652	0.0127	−5.14	circBase	exonic
hsa_circ_0003571	0.0144	−5.09	circBase	exonic
hsa_circ_0005087	0.0161	−5.05	circBase	exonic
hsa_circ_0110719	0.0173	−5.02	circBase	exonic
hsa_circ_0003662	0.0191	−4.98	circBase	exonic
chrM:10847–11,516+	0.0254	−4.86	novel	sense overlapping
chr19:53,883,975–53,888,280+	0.0279	−4.83	novel	intronic
chrM:12376–12,573+	0.0287	−4.81	novel	sense overlapping
hsa_circ_0007843	0.0311	−4.78	circBase	exonic

promotes GC progression by activating c-myc and TGF-β signaling pathways, indicating that it may serve as a potential biomarker for GC.[43] It has been reported that hsa_circ_0009172 serves as a tumor suppressor in gastric cancer by targeting miR-485-3p/NTRK3 axis.[44] Meanwhile, another study uncovered a tumor suppressor function of circEIF4G3 in GC through the regulation of δ-catenin protein stability and miR-4449/SIK1 axis.[45] Study indicated that HOXC6 mRNA may work as a novel biomarker and can be potentially valuable in predicting the prognosis of patients with GC.[46] Hossain et al found that circCEACAM5 and circCOL1A1 might be the potential biomarkers for the diagnosis and treatment of GC.[47] It has been demonstrated that circRNA_102231 is a novel oncogene in GC and acts as a potential biomarker and therapeutic target for GC patients.[48] Zhang et al proved that circNRIP1 sponges miR-149-5p to affect the expression level of AKT1 and eventually acts as a tumor promotor in GC.[49] It has been found that circURI1 directly interacts with heterogeneous nuclear ribonucleoprotein M (hnRNPM) to modulate alternative splicing of genes, involved in the process of cell migration, thus suppressing GC metastasis.[50] However, in the study of CAG, little research has been conducted on mRNAs and circRNAs, and the expression profiles of mRNAs and circRNAs in CAG remain unclear.

In order to investigate the expression profiles of circRNAs in CAG and to screen the immune-related mRNAs and circRNAs in CAG, we conducted high-throughput sequencing in 20 CAG tissues vs 20 CNAG tissues. All of the patients had an endoscopy and a histopathological diagnosis. To explore the potential biological functions of immune-related circRNAs and mRNAs in CAG, immune cell infiltration, WGCNA, GO, and circRNA-mRNA co-expression analysis were performed.

We performed GO enrichment analysis on differentially expressed mRNAs, then top 11 immune-related GO terms of up- and down-regulated mRNAs were listed. The up-regulated mRNAs were mainly enriched in antimicrobial humoral response, viral entry into host cell, neutrophil activation, leukocyte migration etc. It had been reported that 9 times more neutrophils were found in gastric intestinal metaplasia tissues comparing to normal gastric tissue control.[51] The result indicated that neutrophils participate in pathological processes of CAG, which is consistent with our experimental results.

Chronic gastritis is an inflammatory condition of the gastric mucosa, which is mainly characterized by two main features, infiltration of lamina propria by chronic inflammatory cells and glandular atrophy. It is known that inflammatory cellular infiltrate, containing mainly lymphocytes, plasmocytes, and macrophages, is generated in epithelium and lamina propria of the stomach during the development of chronic gastritis.[52] CNAG refers to inflammatory infiltrates confined to

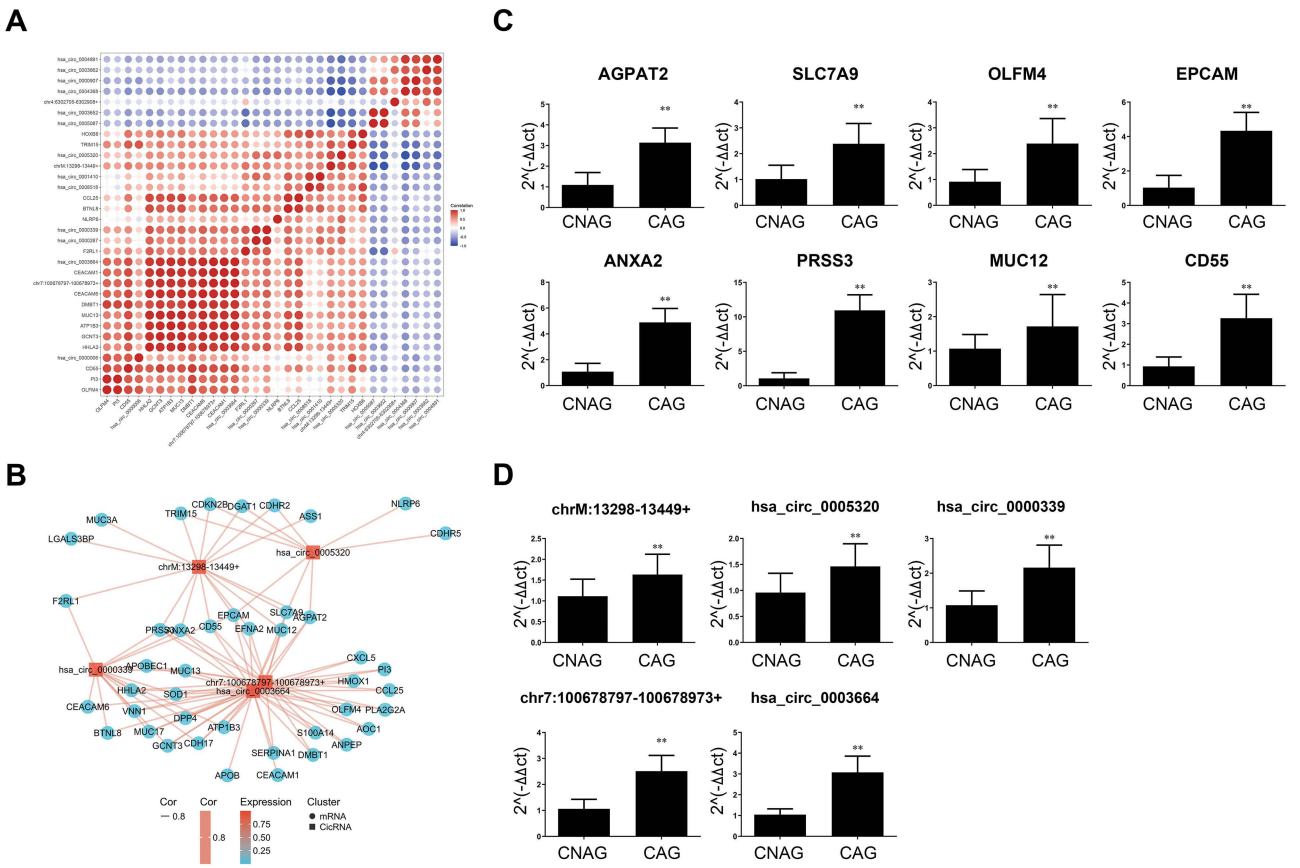

Figure 6 (**A**) Correlation heatmap of immune-related differentially expressed mRNAs and circRNAs. Blue represents negative correlations, and red represents positive correlations. The intensity of the color indicates the strength of the correlation. (**B**) CircRNA-mRNA co-expression network. Red squares represent circRNAs, and blue circles represent mRNAs. (**C**) Expression levels of mRNAs in CAG and CNAG tissues. (**D**) Expression levels of circRNAs in CAG and CNAG tissues. All data are presented as mean ± SD (n = 20). Statistical analysis was performed using unpaired t-tests. **P* < 0.05, ***P* < 0.01.

the superficial layers of the mucosa refers to multifocal glandular atrophy and inflammation observed in antrum and body. We analyzed the DEIRGs of CAG in the previous section, and found these genes involved in neutrophil and lymphocyte functions. Hence, we analyzed the immune cell infiltration in CAG and CNAG, compared with CNAG, CAG had decreased Tcm, NKT, CD8 T cells infiltration and increased neutrophil infiltration. Then we analyzed the percentage of neutrophils, NK cells, Th cells, and Tc cells in CAG by flow cytometry. There was no statistical difference in the percentage of NK cells, Th cells, and Tc cells between the CAG group and CNAG group. The percentage of neutrophils was significantly higher in patients with CAG compared with the CNAG group.

WGCNA was applied to find hub genes which were functionally associated with CAG. WGCNA analysis showed that 9 modules possessed related expression patterns. GO enrichment analysis for black, green and yellow modules containing high mRNA expression was performed. The genes in the yellow module were mainly enriched in the biological processes of extracellular matrix organization, A subset of collagen genes were identified whose expression efficiently distinguished malignant from premalignant lesion in stomach, especially, COL11A1 (collagen type XI alpha 1) and COL1A1 (collagen type I alpha 1) may be a potential biomarker to earlier detect gastric cancer.[53] The genes in the green module were mainly enriched in the biological processes of fat digestion and absorption and organic acid transport, it had been reported that the pathways enriched by upregulated differentially expressed mRNAs were mainly related to fat digestion and absorption, and CYP3A5 (cytochrome P450, family 3, subfamily A, polypeptide 4) had the highest degrees in PPI (protein- protein interaction) network, the result indicated that the expression of CYP3A4 might be related to the potential carcinogenic transformation of CAG to GC. Therefore, CYP3A4 may be biomarkers to predict progression of CAG and poor prognosis of gastric cancer.

By analyzing the sequencing data, we obtained 6,245 circRNA transcripts. The higher numbers of circRNA categories in CAG are exonic, and sense overlapping. There is a study showed that circRNA levels are generally lower than linear RNA levels,[54] and our sequencing results support the idea. The results of transcriptome sequencing revealed that compared with the expression profiles of circRNAs in the CAG and CNAG tissue samples, there were 19 upregulated and 23 downregulated circRNAs in CAG. Most of the differentially expressed circRNAs in CAG have not been given official names, and their functions remain unknown.

In order to further predict the potential functions of circRNAs, we performed the GO analysis of these circRNAs. It had been reported that the levels of both free and total carnitine of the digestive tract malignant tumor patients are lower than the control group.[55] CAG as the precancerous lesion of GC, the upregulated circRNAs in CAG were enriched in carnitine metabolic process. It may therefore be considered that carnitine metabolic play a role in the pathogenesis of CAG, which still needs further verification. One of the significantly enriched GO terms of up-regulated circRNAs in biological process was proteins targeting in Golgi. The results of Yoshimi et al offer an initial preclinical proof of concept for the use of M-COPA as a candidate treatment option for MET-addicted GC, with broader implications for targeting the Golgi apparatus as a novel cancer therapeutic approach.[56] Although there is no direct evidence that Golgi apparatus was linked to CAG, since CAG has been considered to be a precancerous lesion of GC, it cannot be ruled out that Golgi apparatus is involved in the occurrence and development of CAG, which needs to be studied further.

A total of 56 key mRNAs were screened out by WGCNA and Venn diagram, then 16 differentially expressed circRNAs related to key mRNAs were found by calculating PCC values. We selected 5 circRNAs from 16 that were highly related to mRNAs, namely chrM:13298–13449+, hsa_circ_0005320, hsa_circ_0000339, chr7:100678797–-100678973+, hsa_circ_0003664. QRT-PCR was used to verify the expression of these selected circRNAs. We also validated 8 mRNAs (AGPAT2, SLC7A9, OLFM4, EPCAM, ANXA2, PRSS3, MUC12, and CD55) from 56 which were closely related to circRNAs by qRT-PCR. There was research suggested that knocking down OLFM4 may slow the pathological process of IM (Intestinal metaplasia), thus providing putative relevant targets for the treatment of CAG with IM.[57] Other studies showed gradual increase of OLFM4 with further intestinalization of gastric mucosa.[58] There are few new studies on association between other 7 mRNAs, selected 5 circRNAs and CAG. The study speculated that they may be associated with immunity of CAG.

While our study identified key circRNAs and mRNAs associated with CAG, several limitations should be considered. In our study, we did not specifically account for the potential effects of patient age and time since diagnosis on circRNA and mRNA expression profiles. Age is known to influence immune function and inflammatory responses, which may affect biomarker expression in chronic atrophic gastritis. Additionally, the duration of disease progression could impact the molecular landscape, as patients with longer disease duration may exhibit different stages of mucosal atrophy or progression to intestinal metaplasia. These factors could introduce variability in the expression profiles of immune-related circRNAs and mRNAs. Future studies should stratify patients based on age and time since diagnosis to better understand their potential effects on biomarker expression and disease progression in CAG.

Conclusions

In this study, we conducted the first comprehensive analysis of circRNA expression profiles in CAG, revealing novel immune-related mechanisms involving circRNAs in disease progression. We identified 163 key immune-associated mRNAs and, importantly, discovered five circRNAs closely linked to CAG pathogenesis that have not been previously reported. Our study also revealed significant differences in immune cell infiltration, particularly the increased neutrophil infiltration in CAG, suggesting that immune dysregulation plays a central role in disease progression. While validation in larger cohorts is necessary, these findings not only provide new insights into the molecular landscape of CAG but also suggest that the identified circRNAs could serve as potential molecular diagnostic markers or therapeutic targets.

Data Sharing Statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153224, and https://bigd.big.ac.cn/gsa-human/browse/HRA005806.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Jilin University (approval number: 2019082110). All participants were provided with the subject information sheet and gave written informed consent to the study.

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Disclosure

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.

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