Circular RNA expression profiles and identification of hsa_circ_0028381 as a potential biomarker of anti-neutrophil cytoplasmic antibody-associated vasculitis

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Abstract

Objective

Accumulating evidence indicates the role of dysregulated circRNAs in autoimmune diseases. In this study, we investigated their role in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) by analysing the expression profiles of circRNA in plasma of AAV patients and exploring their potential as biomarkers of AAV.

Methods

RNA-sequencing (RNA-seq) was performed to identify the plasma circRNA and mRNA expression profiles from five AAV patients and five healthy controls (HCs). Quantitative reverse-transcription (qRT)-PCR confirmed that hsa_circ_0028381 was confirmed to be significantly upregulated in a validation cohort of 51 AAV patients and 30 HCs and was further verified in other connective tissue diseases (CTDs). The diagnostic value of hsa_circ_0028381 was assessed by receiver operating characteristic (ROC) curve analysis.

Results

RNA expression profiles revealed aberrant expression of 143 circRNAs (62 upregulated and 81 downregulated) and 304 mRNAs (151 upregulated and 153 downregulated) in AAV patients compared to HCs. qRT-PCR verification suggested that hsa_circ_0028381 levels were significantly increased in plasma from AAV patients compared to those in HCs and other CTDs. ROC curve analysis showed has_circ_0028381 had good diagnostic value for distinguishing AAV patients from controls (HCs and other CTDs) with an area under the curve (AUC) of 0.81. In addition, hsa_circ_0028381 was associated with renal involvement. Most importantly, increased baseline levels of hsa_circ_0028381 had predictive value for progression to end-stage renal disease (ESRD).

Conclusion

RNA-seq revealed that circRNAs are aberrantly expressed in the plasma of AAV patients. Hsa_circ_0028381 was implicated as a potential biomarker for AAV diagnosis and renal prognosis.

Key words

anti-neutrophil cytoplasmic antibody, circRNA, has_circ_0028381, biomarker, RNA-seq

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Received on April 26, 2022; accepted in revised form on September 29, 2022.

Funding: this work was supported by theNational Natural Science Foundation of China (NSFC numbers 81800541, 81801598 and 81771730), and the Shanghai Shen Kang Hospital Development Center Clinical Cultivation Project (SHDC12019X05).

Competing interests: none declared.

Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) represents a group of rare and recurrent autoimmune diseases that mainly affect small-to-medium sized blood vessels. AAV can be divided into granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (1, 2). These diseases are characterised by the presence of ANCAs in the serum of most, but not all patients (ANCAs are detected in 70–90% of GPA and MPA cases, and approximately 40% of EGPA) (3). Among which, EGPA shared both necrotising vasculitis and allergic components such as asthma and eosinophilia which is definitely distinct from GPA and MPA (4, 5). Therefore, EGPA was excluded from this research. The diagnosis of AAV is difficult and is often based on a combination of clinical manifestations and laboratory test results (4, 6, 7). The existing diagnostic approach is complex and biopsy may be required for ANCA-negative patients or when the diagnosis is controversial. Due to the heterogeneity in presentation of these diseases, diagnosis is often substantially delayed, leading to deterioration of the clinical outcome. Therefore, a novel biomarker that can be assessed using non-invasive techniques may help to improve AAV diagnosis and the prognosis of patients.

The kidney is one of the most commonly affected organs and closely related to the outcome of AAV (8, 9). Despite improved outcomes of patients with AAV, those with renal impairment remain at a high risk of progression to end-stage renal disease (ESRD) and death (9). Several studies had been conducted to determine risk factors for renal outcome; however, there is still a lack of highly sensitive and specific indicators for predicting renal survival.

Circular RNAs (circRNAs) are a class of noncoding RNAs (ncRNAs) that participate widely in cell growth and development and regulation of gene expression (10). Compared with linear RNAs, circRNAs are generally more stable and conserved due to the presence of a covalently closed-loop structure, which makes them more suitable as biomarkers than linear RNAs.

Many studies have demonstrated the effects of circRNAs on the pathogenesis of human diseases, such as cancers, as well as neurological and autoimmune disorders (11-13). Interestingly, evidence indicates that circRNAs are protected from degradation and exist in a stable form in bodily fluids, especially in plasma; consequently, many plasma circRNAs have been identified as biomarkers for various diseases. Increasing evidence suggests that aberrantly expressed circRNAs can be used as non-invasive indicators for disease diagnosis, activity and prognosis prediction in various autoimmune diseases including primary Sjögren's syndrome (pSS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (14-16). Therefore, we hypothesised that plasma circRNAs represent a promising source of biomarkers for AAV.

In the present study, we first analysed the circRNA expression profiles in the plasma of AAV patients and healthy controls (HCs) by RNA-sequencing (RNA-seq). We then verified four candidates differentially expressed circRNAs in expanded samples from AAV patients, and confirmed significant dysregulation of hsa_circ_0028381 expression. Further investigation of the relationship between hsa_circ_0028381 and the clinical features of AAV patients revealed that hsa_circ_0028381 is associated with renal involvement and poor prognosis. Thus, our findings indicate that hsa_circ_0028381 represents a candidate novel biomarker for AAV diagnosis and renal prognosis.

Material and methods

Patients

We recruited 56 patients with AAV, 27 with SLE and 15 with RA as well as 35 HCs. Patients with AAV conformed to the classification criteria of the American College of Rheumatology (ACR) 1990 and nomenclature of the Chapel Hill 2012 consensus and were followed regularly at Zhongshan Hospital, Fudan University in China (1, 4, 6). The diagnosis of SLE fulfilled the 1997 ACR classification criteria and RA fulfilled the 2010 ACR classifica-

Table I. Demographic, clinical, and laboratory features of all samples.

Characteristic	AAV (n=56)	HC (n=35)	SLE (n=27)	RA (n=15)
Sex (M/F)	28/27	16/19	19/8	13/2
Age (years)	45.45 ± 13.23	42.92 ± 12.97	38.23 ± 15.84	50.20 ± 15.34
Disease duration (months)	8.79 ± 10.38	/	15.34 ± 7.19	13.78 ± 16.53
GPA/MPA	24/32	/	/	/
PR3-ANCA/MPO-ANCA	21/35	/	/	/
BVAS	8.38 ± 6.45	/	/	/
WBC ($\times 10^9$/L)	8.91 ± 7.50	/	7.56 ± 3.25	7.43 ± 4.97
HB (g/L)	118.73 ± 23.54	/	102.4 ± 30.2	120.2 ± 21.8
PLT($\times 10^9$/L)	184.32 ± 47.43	/	132.58 ± 66.82	157.65 ± 41.27
ESR (mm/H)	56.43 ± 37.64	/	37.36 ± 20.25	46.74 ± 32.74
hs-CRP (mg/L)	30.15 ± 41.44	/	10.47 ± 12.43	8.34 ± 10.35
C3 (g/L)	0.89 ± 0.34	/	0.53 ± 1.23	1.01 ± 1.32
C4 (g/L)	0.34 ± 0.19	/	0.09 ± 0.48	0.31 ± 0.53
CH50 (IU/ML)	60.62 ± 19.79	/	32.43 ± 34.64	65.63 ± 23.85
Manifestations				
General	43 (76.8%)	/	/	/
Fever	25 (44.6%)	/	/	/
Fatigue	19 (33.9%)	/	/	/
Arthralgia	25 (44.6%)	/	/	/
Myalgia	15 (26.8%)	/	/	/
Cutaneous	8 (14.3%)	/	/	/
Purpura	6 (10.7%)	/	/	/
Cutaneous ulcers	5 (8.9%)	/	/	/
Livedo reticularis	6 (10.7%)	/	/	/
Eyes	21 (37.5%)	/	/	/
Orbital disease	15 (26.8%)	/	/	/
Uveitis	9 (16.1%)	/	/	/
Scleritis	16 (28.6%)	/	/	/
Retinal vasculitis	10 (17.9%)	/	/	/
ENT	27 (48.2%)	/	/	/
Rhinitis	13 (23.2%)	/	/	/
Paranasal sinus involvement	23 (41.1%)	/	/	/
Otitis media	9 (16.1%)	/	/	/
Sensorineural hearing loss	5 (8.9%)	/	/	/
Chest	47 (83.9%)	/	/	/
Lung nodules	24 (42.9%)	/	/	/
Interstitial lung disease	30 (53.6%)	/	/	/
Pleural effusion	12 (21.4%)	/	/	/
Alveolar haemorrhage	7 (12.5%)	/	/	/
Cardiovascular	3 (5.3%)	/	/	/
Pericarditis	2 (3.6%)	/	/	/
Ischaemic cardiac pain	3 (5.4%)	/	/	/
Kidney	29 (51.8%)	/	/	/
Proteinuria>1+	22 (39.3%)	/	/	/
Haematuria≥10 RBCs/hpf	20 (35.7%)	/	/	/
Renal insufficiency	22 (39.3%)	/	/	/
Creatinine 125–249 μmol/l	14 (25%)	/	/	/
Creatinine 250–499 μmol/l	8 (14.3%)	/	/	/
Nerve system	15 (26.8%)	/	/	/
Cerebrovascular accident	7 (12.5%)	/	/	/
Sensory peripheral neuropathy	10 (17.9%)	/	/	/
Gastrointestinal	2 (3.6%)	/	/	/
Ischaemic abdominal pain	2 (3.6%)	/	/	/
Glucocorticoids (GCs)	35 (62.5%)	/	20 (74.4%)	1 (6.6%)
Immunosuppressants	21 (37.5%)	/	16 (59.3%)	12 (80%)

Data are presented as mean ± standard deviation (SD) or numbers (percentage).
AAV: ANCA-associated vasculitis; HCs: healthy controls; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; M: male; F: female; GPA: granulomatosis with polyangiitis; MPA: microscopic polyangiitis; PR3: proteinase 3; MPO: myeloperoxidase; ANCA: antineutrophil cytoplasmic antibody; BVAS: Birmingham vasculitis activity score; WBC: white blood cell; HB: haemoglobin; PLT: platelet; ESR, erythrocyte sedimentation rate; hs-CRP: high sensitivity C-reactive protein; C3: complement 3; C4: complement 4; CH50: complement haemolysis 50%; ENT: ear, nose, and throat; /: not available or not applicable.

tion criteria (17, 18). Birmingham vasculitis activity score (BVAS) was used to assess the disease activity (19). Patients with estimated glomerular filtration rate (eGFR) <15 mL/min/1.73 m^2 and requiring permanent renal replacement therapy (RRT), or kidney transplantation were classified as having end-stage renal disease (ESRD) (20). Individuals were excluded if they had concurrent infectious diseases or malignancies. The HCs had no history of

autoimmune diseases, infectious diseases or malignancies. This study was approved by the Institutional Review Board of Zhongshan Hospital and all subjects included in this study provided the written informed consent to participate. The demographic characteristics and baseline clinical features of all subjects are summarised in Table I.

Our study was conducted in three stages. In the first (screening) stage, plasma samples obtained from five AAV patients (3 GPA and 2 MPA) and five HCs were selected for RNA-sequencing (RNA-Seq). In the second stage, the expression of four candidate circRNAs in plasma was verified by quantitative reverse-transcription (qRT)-PCR in the remaining 51 AAV patients and 30 HCs. In the third stage, the expression level of hsa_circ_0028381 was further examined in 27 SLE patients and 15 RA patients and ROC curve analysis was performed to verify its diagnostic power. The study design is shown in Figure 1.

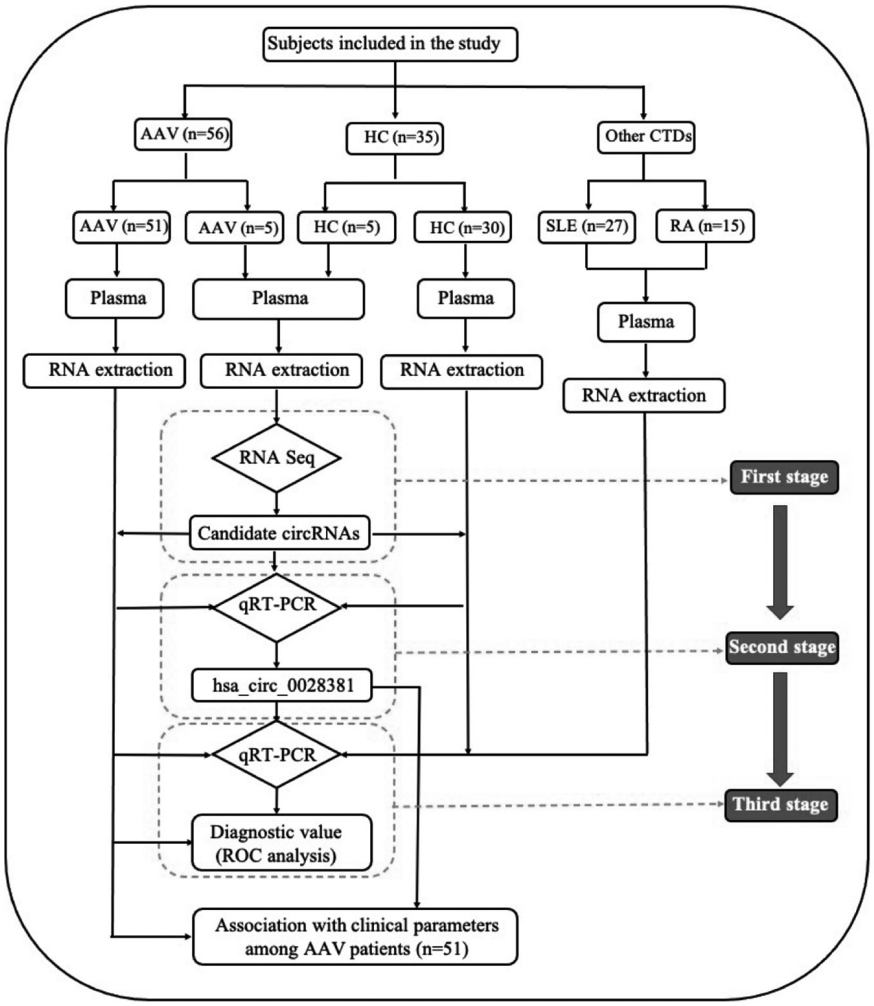

Fig. 1. Study design.
The study included 56 patients with AAV, 35 HCs and 42 patients with other CTDs (27 SLE and 15 RA). In the first stage, plasma from five patients with AAV and five age- and sex-matched HCs were selected for RNA-Seq. In the second stage, candidate circRNAs were validated by qRT-PCR in the remaining 51 AAV patients and 30 HCs. In the third stage, hsa_circ_0028381 was further verified by qRT-PCR in other CTDs and ROC curve analysis was used to assess its diagnostic value. Furthermore, the association of hsa_circ_0028381 with clinical parameters among AAV patients (n = 51) was also evaluated.
AAV: ANCA-associated vasculitis; HCs: healthy controls; RNA-Seq: RNA-sequencing; qRT-PCR: quantitative reverse transcription real-time PCR; CTDs: connective tissue diseases; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; ROC: receiver operating characteristic.

Plasma preparation and Total RNA extraction from plasma

Blood samples (approximately 10 ml) were collected into EDTA-treated tubes and immediately centrifuged at 3,000 ×*g* for 15 min at 4°C. The plasma was isolated and stored at -80°C prior to RNA extraction. Total RNA was extracted from plasma samples using TRIzol LS (Invitrogen, Carlsbad, CA, USA). Subsequently, the quality of the isolated RNA samples was assessed by an Agilent Bioanalyzer 2100 (Agilent technologies) and purity were measured using a NanoDrop spectrophotometer-2000 (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was stored at -80˚C for further analysis.

RNA sequencing

RNA libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina, cat. no. RS-122-2301). All transcriptomes were pooled and merged to generate a final transcriptome using Cuffmerge (Cufflinks 2.0). CircRNAs were identified using CIRI (v. 2.0.3) and expression levels were calculated using the RPM (spliced reads per million mapped) algorithm. For mRNAs, the FPKM (fragments per kilobase of exon per million reads) of each gene was calculated using Cufflinks, and the read counts of each gene were obtained by HTSeq-count. The DESeq (2012) R package was used to analyse the differential expression of circRNAs and mRNAs. The thresholds for differentially expressed genes were set at false discovery rate (FDR) <0.05, and fold change (FC) >2. The RNA sequencing process and analyses were performed by OE Biotech Co., Ltd. (Shanghai, China).

qRT-PCR

Total RNA extracted from plasma was reverse-transcribed to synthesise cDNA using random primers with the PrimeScript RT Reagent Kit (Takara, Shiga, Japan). qRT-PCR analysis was performed with the SYBR Premix Ex Taq II (Takara, Shiga, Japan) on a CFX 96 Connect system (Bio-Rad, USA). The relative expression of circRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal control. The primers used are as follows: β-actin-F: GTGGCCGAGGACTTTGATTG; β-actin-R: CCTGTAACAACGCATC-TCATATT; hsa_circ_0007992-F: ACGCGCTTCTGCAATCCAAG; hsa_circ_0007992-R: TCCATTATC-

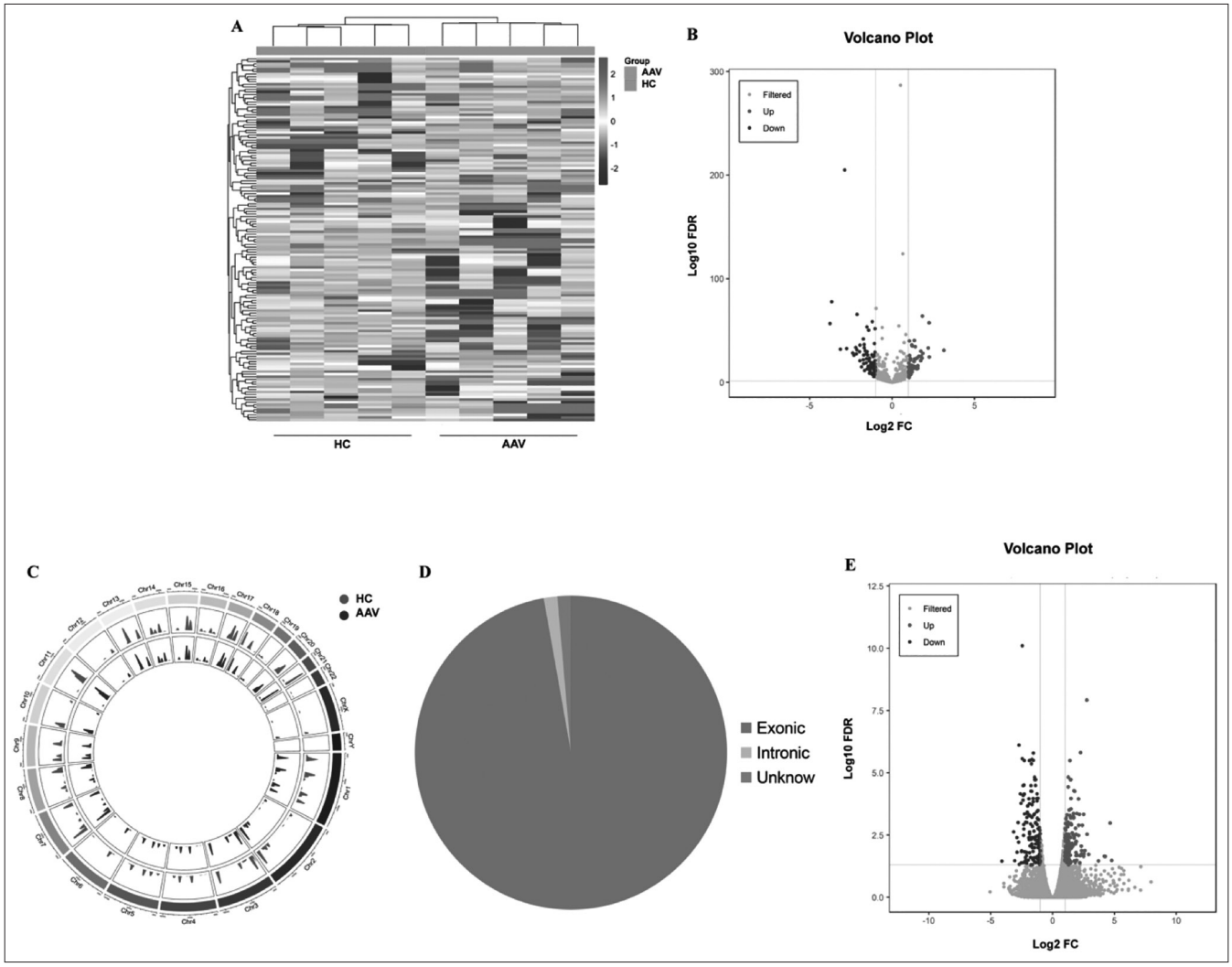

Fig. 2. Differential expression profile of circRNAs and mRNAs in plasma from AAV patients and HCs.
A: The heat map shows the differentially expressed circRNAs detected by RNA-Seq. Red represents relatively high expression and blue represents relatively low expression. White represents no obvious change.
B: Volcano plots shows the significantly dysregulated circRNAs between AAV patients and HCs. The red and blue spots indicate the significantly upregulated and downregulated circRNAs, respectively (FC >2 and FDR <0.05).
C: Circos plot shows the locations and expression levels of dysregulated circRNAs on human chromosomes. The outermost circle is a chromosome map of the human genome. The middle circle represents circRNAs of HCs and the innermost circle represents was circRNAs from AAV patients.
D: Percentage of significantly dysregulated circRNAs from different genomic regions.
E: Volcano plots shows the aberrantly expressed mRNAs between AAV patients and HCs. The red and blue spots indicate the significantly upregulated and downregulated mRNAs, respectively (FC >2 and FDR <0.05).
AAV: ANCA-associated vasculitis; HCs: healthy controls; RNA-Seq: RNA sequencing; FC: fold-change; FDR: false discovery rate.

TTCTCGACTCTTTCTGGA; hsa_circ_0028381-F: GAAATCGGGCCTGGACCAGA; hsa_circ_0028381-R: TGTCTTCGGTCAGCCTGCAA; hsa_circ_0000886-F: GGGAGCACCAAGGTCTTTGTC; hsa_circ_0000886-R: ATCAGTGTGGCCACCAGGTC; hsa_circ_0001725-F: TGCCCGAGGACTGGAAGAAG; hsa_circ_0001725-R: AATACGGCTGTGCGGCATTG.

Bioinformatics analysis

Hierarchical clustering analysis was used to evaluate the variations in circRNAs between AAV patients and HCs. Volcano plot filtering analysis was used to present the aberrant expression of circRNAs. Gene Ontology (GO) analysis was performed to clarify the biological significance of differentially expressed genes (DEGs). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was used to predict significant pathways involving the DEGs.

Construction of circRNA-miRNA-mRNA interaction networks

First, the Pearson coefficients of dysregulated circRNAs and mRNAs were calculated based on the gene expression levels. Next, we used the correlation coefficients to identify circRNA-mRNA co-expression pairs. Then, the miRanda-3.3a database was used to predicate the miRNA targets of the circRNAs and mRNAs in the identified co-expression pairs.

Ultimately, by calculating the intersection of the predicted miRNAs for circRNAs and mRNAs, we established a circRNA-miRNA-mRNA interaction network.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism (v. 6.0; GraphPad Software, CA, USA) and SPSS (v. 25.0; SPSS, Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD), and categorical variables were presented as the number and percentage. Differences in circRNA levels among the groups were analysed using Student's *t*-test or the Mann-Whitney test. Receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic value of selected circRNAs. The odds ratio (OR) was calculated using contingency tables and chi-square tests. $p<0.05$ was set as the threshold for statistical significance.

Results

Expression profile of circRNAs in plasma from AAV patients

We performed RNA-Seq to analyse the differential expression of circRNAs in plasma samples from five AAV patients and five HCs. Differentially expressed circRNAs between AAV patients and HCs were visualised by hierarchical clustering (Fig. 2A) and volcano plots (Fig. 2B). In total, 639 circRNAs were detected, of which 143 were differentially expressed. Compared with HCs, 62 circRNAs were significantly upregulated and 81 circRNAs were downregulated in AAV patients (FC >2.0 and FDR <0.05). Moreover, the differentially expressed circRNAs were located on all chromosomes, including the X and Y sex chromosomes, and most (97.2%) were derived from exons (Fig. 2C and 2D). In addition, a total of 304 mRNAs were differentially expressed using the same cut-off criteria (151 upregulated and 153 downregulated). Figure 2E shows a volcano plot of the DEGs. These data indicate that circRNAs have a different expression pattern in AAV patients compared with that in HCs.

The bioinformatics analysis of differentially expressed circRNAs

To clarify the biological functions associated with the host genes of differentially expressed circRNAs, we performed GO and KEGG pathway analyses. The top 10 significantly enriched GO processes were identified for the biological process (BP), molecular function (MF) and cellular component (CC) categories (Fig. 3A). In the BP category, the top three processes were involved in extracellular exosome, positive regulation of viral genome replication and cellular response to hypoxia. In the MF category, neurotransmitter receptor metabolic process, ciliary basal body-plasma membrane docking and autophagosome assembly ranked as the top three terms. As for CC category, the GO terms were significantly enriched in anterograde dendritic transport of the mitochondrion and negative regulation of T cell proliferation. KEGG pathway analysis was conducted to identify the key signalling pathways associated with the dysregulated circRNAs (Fig. 3B). The top five pathways were base excision repair, complement and coagulation cascades, aldosterone synthesis and secretion, nucleotide excision repair and pyrimidine metabolism.

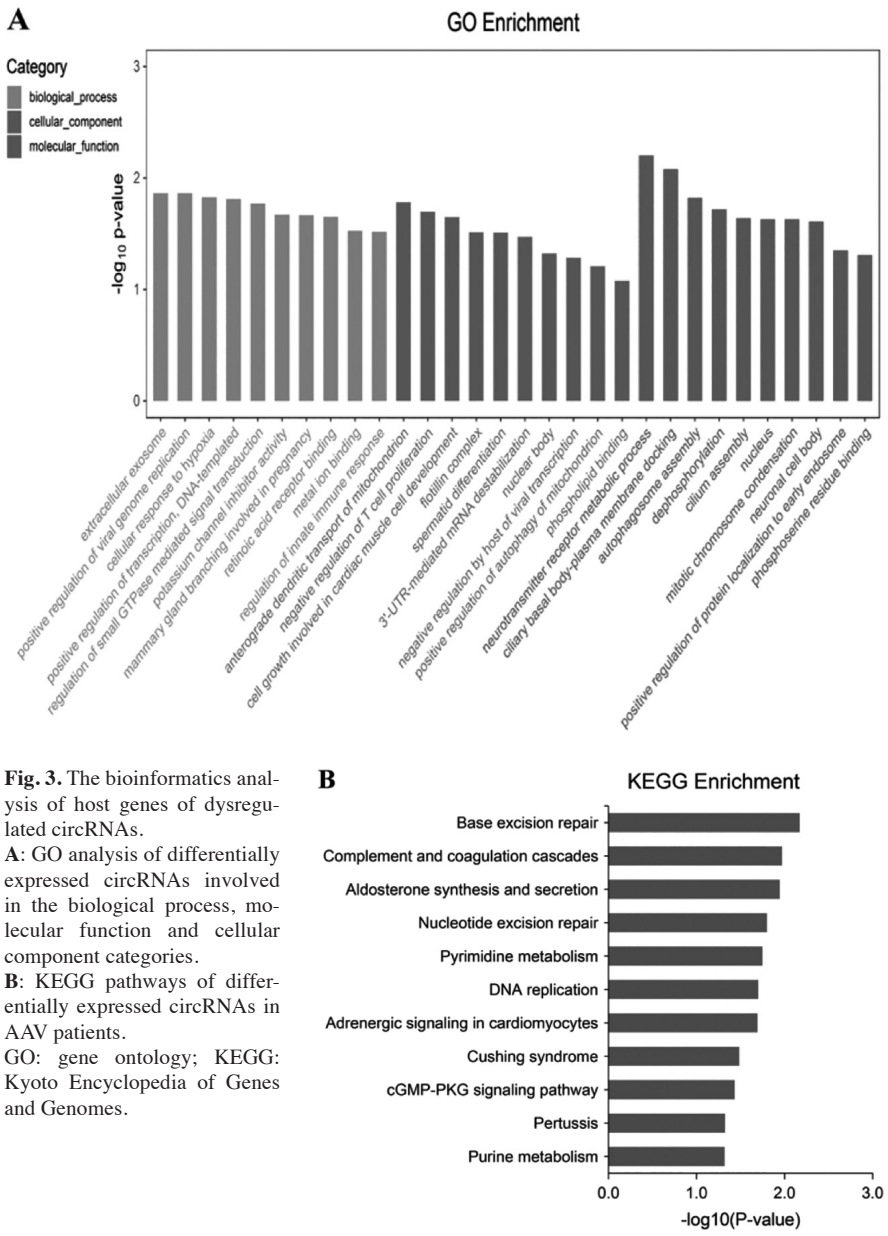

Fig. 3. The bioinformatics analysis of host genes of dysregulated circRNAs.
A: GO analysis of differentially expressed circRNAs involved in the biological process, molecular function and cellular component categories.
B: KEGG pathways of differentially expressed circRNAs in AAV patients.
GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Construction of a ceRNA network

There is strong evidence that circRNAs function as miRNA sponges to regulate target gene expression (10, 21). In this study, we constructed a ceRNA network to explore the interactions between circRNA, miRNA and mRNA. First, we selected the top 20 dysregulated cir-

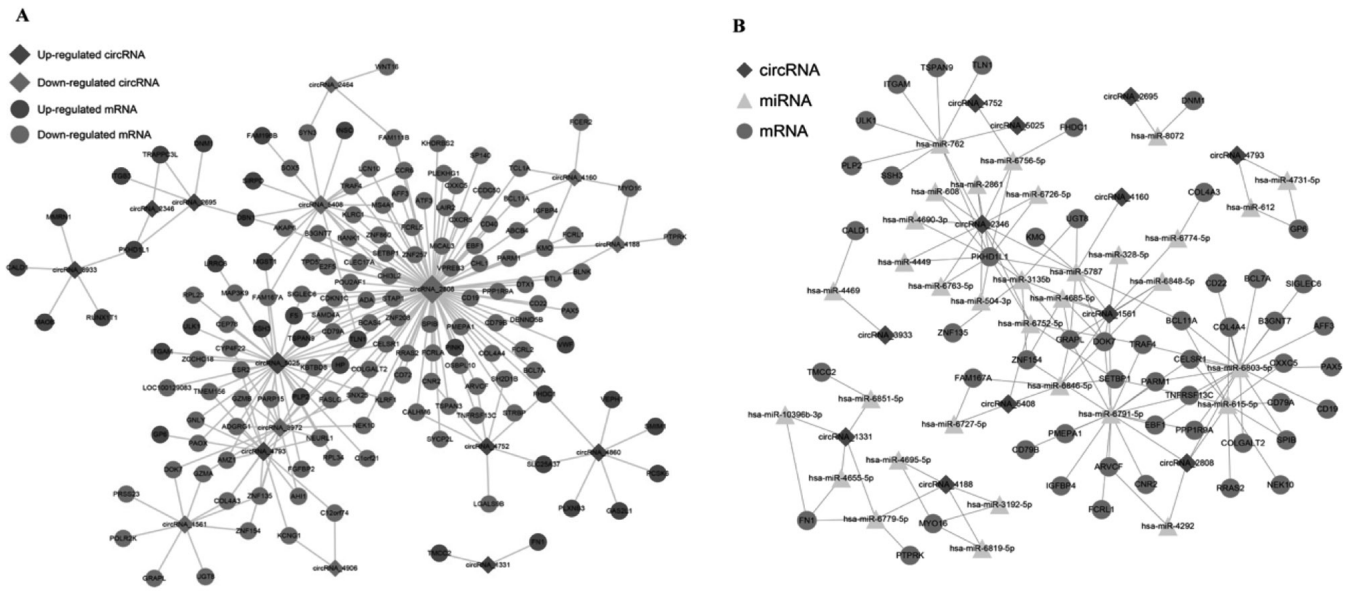

Fig. 4. The interaction network of circRNAs, miRNAs and mRNAs.
A: CircRNA and mRNA co-expression networks. Rhombic nodes represent circRNAs, circular nodes represent mRNAs, red represents upregulation, and blue represents downregulation.
B: Predicated target miRNAs of dysregulated circRNAs (top 10 upregulated and downregulated circRNAs) and their co-expressed mRNAs. Rhombic nodes represent circRNAs, triangular nodes represent target miRNAs, and circular nodes represent co-expressed mRNAs of circRNAs.

cRNAs (10 upregulated and 10 downregulated) and established a circRNA-mRNA co-expression network of 226 matched circRNA-mRNA pairs (Fig. 4A). We then used the miRanda database to predict the miRNAs that bind to these circRNAs and mRNAs. The intersection of the miRNAs predicted for circRNAs and mRNAs were identified as those that can simultaneously binds with circRNAs and mRNAs. A total of 12 circRNAs, 51 mRNAs, and 33 predicted miRNAs were contained in the resulting ceRNA network (Fig. 4B).

Hsa_circ_0028381 might be a new diagnostic biomarker of AAV

To identify potential biomarkers of AAV, the 4 most significantly upregulated circRNAs (circRNA_4752, circRNA_1331, circRNA_2695 and circRNA_4793, corresponding to CircBase ID hsa_circ_0007992, hsa_circ_0028381, hsa_circ_0000886 and hsa_circ_0001725, respectively) were selected for further validation by qRT-PCR. The expression trends of all four circRNAs were consistent with the RNA-seq data, whereas hsa_circ_0028381 expression levels alone differed significantly between AAV patients and HCs (p<0.05) (Fig. 5).

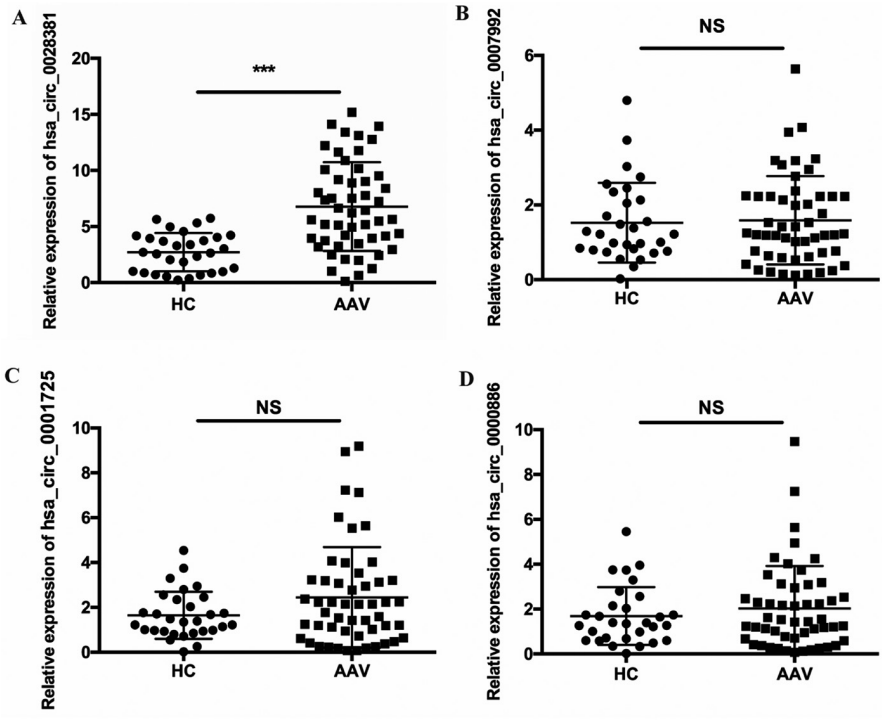

Fig. 5. Validation of four selected circRNAs by qRT-PCR.
Relative expression levels of hsa_circ_0028381 (**A**), hsa_circ_0007992 (**B**), hsa_circ_0001725 (**C**) and hsa_circ_0000886 (**D**) were determined in the plasma of 51 AAV patients and 30 HCs by qRT-PCR.
qRT-PCR: quantitative reverse transcription real-time PCR; AAV: ANCA-associated vasculitis; HCs: healthy controls.

To verify the disease specificity of hsa_circ_0028381 identified in AAV patients, we enrolled patients with RA (n=15) and SLE (n=27) as non-AAV CTD controls. As shown in Figure 6A, there were no significant differences in hsa_circ_0028381 expression levels among the HCs and patients with SLE and RA. Moreover, we performed ROC curve analysis to evaluate the diagnostic ef-

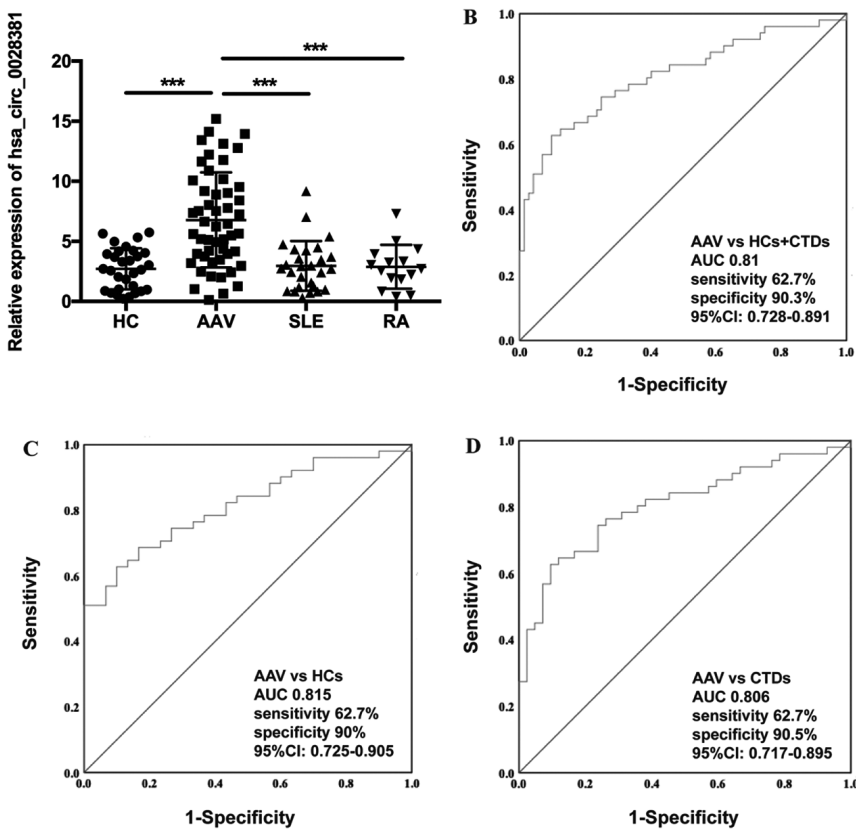

Fig. 6. Plasma hsa_circ_0028381 as a diagnostic marker for AAV.
A: Relative expression levels of hsa_circ_0028381 in plasma among different groups.
B: ROC curves of hsa_circ_0028381 for distinguishing AAV from controls (HCs and other CTDs).
C-**D**: ROC curves of hsa_circ_0028381 for distinguishing AAV from HCs (**B**) and patients with other CTDs (**C**).
AAV: ANCA-associated vasculitis; HCs: healthy controls; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; ROC: receiver operating characteristic; CTD: connective tissue diseases; AUC: the area under the ROC curve.

ficacy of hsa_circ_0028381 in AAV. Hsa_circ_0028381 had an AUC of 0.81 for discriminating AAV patients from controls (HCs and other CTDs) with a sensitivity of 62.7% and specificity of 90.3% (Fig. 6B). When discriminating AAV patients from HCs, the AUC for hsa_circ_0028381 was 0.815 with sensitivity of 62.7% and specificity of 90% (Fig. 6C). When hsa_circ_0028381 was used to discriminate AAV patients from other CTD controls, the AUC was 0.806 with sensitivity of 62.7% and specificity of 90.5% (Fig. 6D). These data indicated that hsa_circ_0028381 shows good diagnostic value for AAV and represents a potential biomarker for AAV diagnosis.

Association of hsa_circ_0028381 expression with the clinical characteristics of AAV

Next, we explored the relationship between hsa_circ_0028381 and the clinical manifestations, laboratory test results and medical treatment (Table II). Compared with patients without renal manifestations, hsa_circ_0028381 expression was upregulated in patients with renal involvement. However, there was no significant difference in hsa_circ_0028381 expression levels between patients with and without other clinical symptoms.

Moreover, we found that increased hsa_circ_0028381 expression in AAV patients was closely related to low haemoglobin, low C3 and positive urine occult blood. However, in this study, we did not find an association between hsa_circ_0028381 and the use of glucocorticoids (GCs) or immunosuppressants for the treatment of AVV.

Hsa_circ_0028381 predicated poor renal prognosis

Having found that increased hsa_circ_0028381 was correlated with renal involvement, we next investigated the potential of hsa_circ_0028381 to predict renal outcome. All patients with renal involvement were without ESRD at the time of sample collection, which was defined as the baseline. The average serum creatinine concentration at baseline was 158.9 μmol/L (range, 65–342 μmol/L) and the mean eGFR (CKD-EPI) was 56.3 ml/min/1.73 m^2 (range, 37–92 ml/min/1.73m^2). All patients had microhaematuria and 76% of patients had proteinuria (mean 1.2 g/day; range, 0.7–2.3 g/day). During the mean follow-up of 1.8 years (range 0.8–3.7 years), 10 of 29 patients had ESRD (9 patients required acute dialysis and 1 patient received a renal transplant).

We then divided the patients into two subgroups according to whether they progressed to ESRD. We found that patients who progressed to ESRD exhibited higher levels of hsa_circ_0028381 than those without at baseline (Fig. 7A). Moreover, we performed ROC curve analysis to assess the value of hsa_circ_0028381 in differentiating between patients who progressed to ESRD and those who did not. Using a cut-off value of 9.11, hsa_circ_0028381 predicted ESRD with an AUC value of 0.857 (sensitivity 90% and specificity 82.6%) (Fig. 7B). In addition, patients with higher hsa_circ_0028381 levels exhibited a higher risk of progression to ESRD (OR = 33.75, 95% CI 3.245–351.052, p=0.001) (Fig. 7C). These data indicated that hsa_circ_0028381 at baseline is a valuable predictor of ESRD in AAV patients with renal involvement.

Discussion

AAV is a chronic and recurrent autoimmune disease that can affect multiple organ systems (22). A timely diagnosis can improve the prognosis of AAV patients. However, in clinical practice, the diagnosis of AAV is hampered and often delayed due to the complex and silent nature of the disease. To date, no single diagnostic indicator has been identified with high sensitivity and specificity. Thus, a novel surrogate biomarker for accurate diagnosis of AAV is urgently required. CircRNAs represent impor-

Table II. Hsa_circ_0028381 levels in AAV patients according to clinical features, laboratory test results and medical treatment.

Clinical features	All patients (n=51)					GPA (n=21)					MPA (n=30)				
	Presence of clinical features		Absence of clinical features		*p*	Presence of clinical features		Absence of clinical features		*p*	Presence of clinical features		Absence of clinical features		*p*
	n	circRNA expression levels	n	circRNA expression levels		n	circRNA expression levels	n	circRNA expression levels		n	circRNA expression levels	n	circRNA expression levels	
Fever	22	6.26±4.71	29	7.17±3.31	0.447	10	7.46±4.60	11	8.03±3.34	0.747	12	5.26±4.77	18	6.64±3.27	0.355
ENT	24	5.72±4.49	27	7.71±3.21	0.805	16	5.30±3.80	5	8.6±3.74	0.104	8	6.59±5.85	22	7.51±3.14	0.682
Ophthalmic	18	6.82±3.94	33	6.75±4.02	0.952	14	6.85±4.13	7	6.60±4.70	0.901	4	6.72±3.72	26	6.79±3.93	0.971
Rash	8	8.48±2.83	43	6.40±4.11	0.101	4	8.69±2.54	17	5.39±4.65	0.193	4	8.29±3.49	26	7.16±3.58	0.559
Arthralgia	13	8.05±3.83	38	6.34±3.95	0.182	5	7.87±3.05	16	4.95±4.30	0.178	8	8.17±4.45	22	7.35±3.44	0.599
Lung	43	6.56±3.83	8	7.94±4.69	0.454	17	5.71±3.66	4	8.16±3.27	0.235	26	7.12±4.17	4	7.70±4.80	0.800
Renal	29	8.72±3. 58	22	4.22±2.86	**<0.001**	11	8.48±3.61	10	3.91±2.40	**0.003**	18	8.87±3. 66	12	4.48±3.27	**0.002**
Neurologic	15	6.76±4.69	36	6.78±3.68	0.985	5	6.37±4.23	16	6.81±4.45	0.848	10	6.95±5.12	20	6.76±3.06	0.916
Cardiovascular	3	6.56±3.90	48	6.79±4.00	0.930	0	NA	21	6.56±4.11	NA	3	6.56±3.90	27	6.97±3.99	0.869
Gastrointestinal	2	5.64±5.03	49	6.82±3.97	0.796	0	NA	21	6.39±4.36	NA	2	5.64±5.03	28	7.15±3.70	0.588
Laboratory test															
WBC<3.5×10^9/L	8	6.82±4.47	43	6.77±3.91	0.972	5	6.69±4.42	16	5.18±4.55	0.522	3	7.04±5.54	27	7.71±3.21	0.750
HB<115g/L	20	9.89±3.58	31	4.77±2.71	**0.000**	8	9.58±3.71	13	5.52±3.00	**0.012**	12	10.10±3.64	18	4.22±2.42	**0.000**
PLT<125×10^9/L	12	7.53±4.36	39	6.55±3.86	0.456	7	8.35±4.07	14	5.50±4.14	0.151	5	6.39±4.95	25	7.13±3.64	0.697
ESR>34mm/H (female)															
>28mm/H (male)	38	7.34±3.92	13	6.58±4.00	0.560	17	5.96±4.53	4	7.17±4.37	0.635	21	7.09±3.55	9	7/41±3.99	0.829
Urine protein	22	7.46±3.60	29	6.26±4.20	0.286	15	6.91±3.27	6	6.64±4.50	0.880	7	8.65±4.24	23	6.16±4.21	0.182
Urine occult blood	20	8.74±3.77	31	5.49±3.22	**0.001**	10	8.72±3.51	11	5.26±3.81	**0.044**	10	8.82±2.84	20	5.62±4.12	**0.036**
hs-CRP>3mg/L	18	7.22±4.78	33	6.53±3.48	0.595	11	6.34±4.96	10	6.78±4.50	0.833	7	8.62±4.48	23	6.43±3.05	0.148
C3<0.7g/L	28	8.24±4.57	23	4.99±1.97	0.002	12	8.12±3.96	9	4.87±1.91	0.022	16	8.29±5.11	14	5.07±2.06	0.031
C4<0.1g/L	17	8.15±4.61	34	6.09±3.46	0.080	9	8.55±4.97	12	6.26±4.04	0.259	8	7.70±4.47	22	6.00±3.20	0.258
CH50<50IU/ml	20	7.31±4.73	31	6.43±3.41	0.475	10	8.00±5.47	11	6.64±4.17	0.527	10	6.63±4.04	20	6.32±3.03	0.813
Treatment															
Glucocorticoids (GCs)	35	6.39±4.31	16	7.63±2.99	0.238	15	6.15±3.91	6	7.12±2.96	0.594	20	6.56±4.69	10	7.94±3.11	0.407
Immunosuppressants	21	7.13±4.58	30	6.23±2.76	0.385	10	7.70±5.06	11	6.56±3.54	0.561	11	6.64±4.63	19	6.15±2.82	0.719

Data are presented as mean ± standard deviation (SD).
ENT: ear, nose, and throat; WBC: white blood cell; HB: haemoglobin; PLT: platelet; ESR: erythrocyte sedimentation rate; hs-CRP: high sensitivity C-reactive protein; C3: complement 3; C4: complement 4; CH50: complement haemolysis 50%; NA: not applicable.

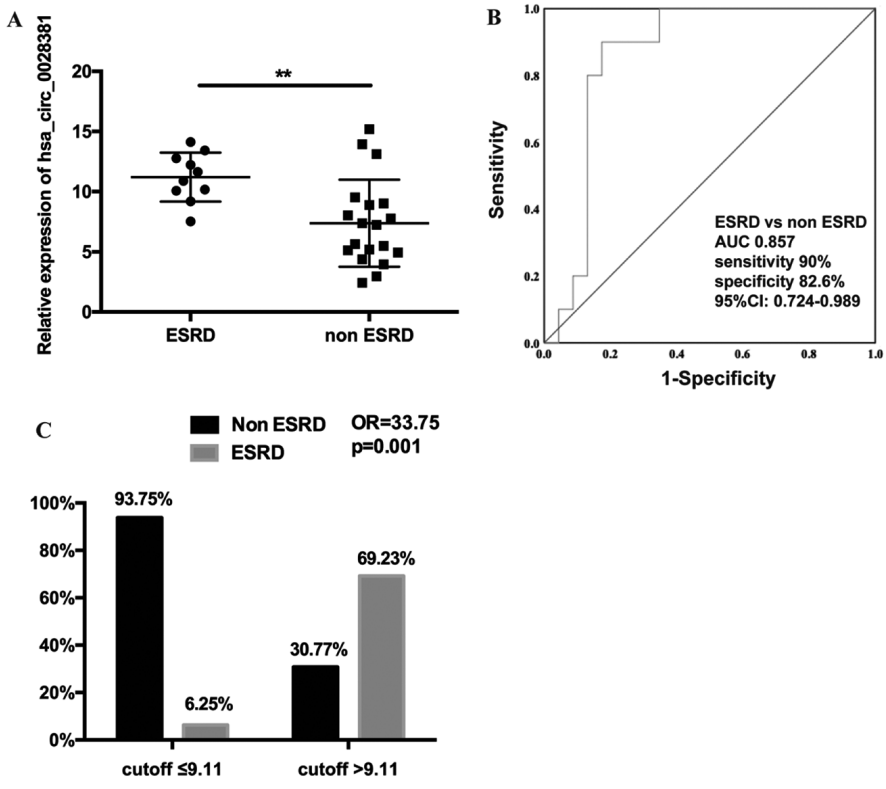

Fig. 7. Clinical significance of hsa_circ_0028381 for predicting ESRD in AAV patients with renal involvement.
A: Comparison of baseline hsa_circ_0028381 expression in AAV patients with renal involvement who developed ESRD and those who did not.
B: ROC curve of hsa_circ_0028381 for discriminating AAV patients with renal involvement who developed ESRD from those who did not.
C: OR of ESRD based on hsa_circ_0028381 level.
ESRD: end-stage renal disease; AAV: ANCA-associated vasculitis; ROC: receiver operating characteristic; AUC: area under the ROC curve; OR, odds ratio.

tant members of the noncoding RNA family that were initially considered to be the product of transcriptional noise (23). However, recent studies have indicated that circRNAs have numerous biological functions, acting as miRNA sponges, interacting with RNA binding proteins (RBPs), regulating parental gene transcription and direct translation of genes (24, 25). Compared with miRNAs and lncRNAs, circRNAs are more widely expressed, highly conservative and more stable due to their unique 'head-to-tail' structure (26-28). Studies have shown that circRNAs are aberrantly expressed in tissues and peripheral blood and have been confirmed as potential biomarkers for the diagnosis and pathogenesis for a number of diseases (29-31). However, aberrant expression of circRNAs and their roles in AAV has never been investigated. In this study, we explored the expression profiles of circRNAs and their potential roles as biomarkers of AAV.

In current study, we comprehensively analysed the expression profiles of circRNAs and mRNAs from the plasma of AAV patients using RNA-Seq. A total of 144 circRNAs were found to be dysregulated (63 upregulated and 81 downregulated) in AAV patients compared with the HCs, of which 97.2% originated from exons. Furthermore, GO analysis indicated that the differentially expressed circRNA-derived genes were enriched in immune response processes, such as regulation of innate immune responses and T cell proliferation. In addition, KEGG analysis indicated that the host genes of circRNAs were dramatically enriched in complement and coagulation cascades, which plays a critical role in the pathogenesis of AAV (32, 33). Thus, we speculate that these aberrantly expressed circRNAs participate in the pathogenesis of AAV by regulating immune responses and the complement system.

CircRNAs can act as miRNA "sponges" to regulate target genes and their involvement in diverse autoimmune disorders has been confirmed (21). However, the existence of ceRNA networks in AAV remains to be established. Dissecting the ceRNA network in AAV may help us better understand the role of circRNAs and expand our knowledge of epigenetic regulation in the pathogenesis of AAV. Therefore, we constructed a circRNA-miRNA-mRNA network based on RNA-Seq data and a prediction database. The results revealed a complex network in which one circRNA can target multiple mRNAs and one mRNA can also be targeted by various circRNAs, indicating that circRNAs participate in the pathogenesis of AAV via numerous pathways. However, this is a predicted network that warrants further validation.

In this study, we investigated the potential of circRNAs to serve as biomarkers of AAV. We validated four candidate circRNAs in plasma samples of AAV patients and HCs as well as patients with other CTDs. Hsa_circ_0028381 was significantly upregulated in AAV patients compared to HCs and patients with other CTDs, indicating the function of hsa_circ_0028381 in the pathogenesis of AAV. Moreover, we showed that hsa_circ_0028381 had good diagnostic value (AUC 0.81) for discriminating AAV patients from controls (HCs and other CTDs) with high specificity. Thus, we identified hsa_circ_0028381 as a potential diagnostic biomarker for AAV.

We also investigated the potential association of hsa_circ_0028381 expression with the clinical features of AAV patients. Laboratory tests suggested that hsa_circ_0028381 was negatively related to haemoglobin and C3 levels. The alternative complement pathway is activated and plays a central role in AAV pathogenesis (32, 33). Furthermore, lower serum C3 levels at diagnosis are predictive of shorter survival and C3d deposition in renal biopsies has been associated with crescent formation (34, 35). Moreover, high hsa_circ_0028381 expression was associated with urine occult blood positivity. In terms of clinical manifestations, increased hsa_circ_0028381 expression was associated the renal involvement. Thus, our results indicate the involvement of hsa_circ_0028381 in renal pathology and prognosis.

Renal involvement occurs in 75–90% of AAV patients and is associated with a poor prognosis with high mortality, particularly among those with ESRD (36). The ability to predict patients at high risk of ESRD will aid in the determining the most appropriate treatment for these individuals. In previous studies, decreased C3 levels at diagnosis was identified as a predictor of poor renal outcome (20). In light of our previous discovery that hsa_circ_0028381 levels are related to renal involvement and negatively related to C3 levels, we further investigated the potential of hsa_circ_0028381 levels as a predictor of renal outcome. As expected, higher hsa_circ_0028381 expression was shown to be predictive of the occurrence of progression to ESRD in patients with renal involvement. Thus, these findings implicate hsa_circ_0028381 as a potential biomarker of renal prognosis.

Studies have shown that the regulatory effects of circRNAs on host genes are mediated via various mechanisms (37). *HECTD4*, the host gene of hsa_circ_0028381, has not been well illustrated, although some studies have indicated its regulatory function in glucose metabolism and ubiquitin protein ligase activity (38, 39). Based on the GO analysis of dysregulated circRNAs, we speculated that hsa_circ_0028381 might be involved in the pathogenesis of AAV by regulating the glucose metabolism and ubiquitination process of immune cells. However, this hypothesis needs to be deeply investigated in the future. Moreover, the pathogenesis mediated by hsa_circ_0028381 in AVV also should be investigated in the future.

Although the sample size in the current study is relatively small and all samples were from the same hospital, our study provides evidence of the clinical diagnostic value of hsa_circ_0028381 for AVV, especially for the prognosis of patients with renal involvement. Thus, our study provides a basis and reference for future large-sample studies to verify its clinical applicability among populations of different ethnicities and regions.

Conclusions

In conclusion, this is the first study to demonstrate the aberrant expression of numerous circRNAs in AAV patients. Furthermore, our findings implicate hsa_circ_0028381 as a potential biomarker for AAV diagnosis and predica-

tion of renal prognosis. Thus, our study provides a foundation for future research on the role of circRNA in AAV. However, further studies are required to determine the clinical significance of hsa_circ_0028381 and other circRNAs as well as the underlying mechanisms to support their therapeutic potential in AAV.

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