Molecular Therapy
Nucleic Acids
Original Article

circHLA-C Plays an Important Role in Lupus Nephritis by Sponging miR-150

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Circular RNAs (circRNAs) participate in the pathogenesis of various diseases by sponging microRNAs (miRs). However, the roles of circRNAs remain unreported in glomerular diseases. We previously reported that miR-150 positively correlated with renal chronicity index in patients with lupus nephritis (LN). We aimed to investigate renal circRNA profiling and the interaction between circRNAs and miR-150 in LN patients. Six renal biopsies from untreated female patients with LN class IV and five normal kidney tissues from urology patients were used for circRNA sequencing. 171 circRNAs with 2-fold differential expression were identified in LN compared with normal control. Ten selected circRNAs were validated by real-time qPCR, and seven circRNAs showed the same significant increases as the sequencing results. circHLA-C positively correlated with proteinuria ($R = 0.92$, $p < 0.01$), serum creatinine ($R = 0.76$, $p = 0.08$), renal activity index ($R = 0.88$, $p < 0.05$), and crescentic glomeruli ($R = 0.93$, $p < 0.01$). Renal circHLA-C increased 2.72-fold, and miR-150 decreased 66% in LN compared with normal control ($p < 0.05$). Bio-informatic analysis predicted miR-150 was regulated by circHLA-C and displayed one perfect match seed between circHLA-C and miR-150. The renal miR-150 showed a tendency of negative correlation with circHLA-C in LN patients. In conclusion, circHLA-C may play an important role in the pathogenesis of lupus nephritis by sponging miR-150.

INTRODUCTION

Despite improvements in renal outcomes in lupus nephritis (LN), 10% of LN patients still progress to end-stage renal disease (ESRD).[1] LN class IV particularly has the highest risk of ESRD, with a 15-year risk of 44% during the 2000s, and is the most common class of LN.[2] The persistent occurrence of ESRD is considered to be associated with the limited understanding of pathogenesis of LN and the absence of effective therapeutic targets.

Circular RNAs (circRNAs) are a class of newly identified non-coding RNAs and are produced from precursor mRNA backsplicing by covalently closed, single-stranded RNA circles at the junction site of 3′5′-phosphodiester bond.[3] circRNAs have been demonstrated to function as microRNAs (miRs) sponges, regulate transcription of genes, and are more stable than linear RNAs.[3-5] In addition, circRNAs also participate in the translation of proteins.[6,7] Previous studies have reported that circRNAs possess cell-type-specific and tissue-type-specific expression[5,8,9] and are involved in pathogenesis of various human diseases such as cardiovascular diseases,[10-13] neural systemic diseases,[14-17] osteoarthritis,[18] diabetes mellitus,[19] and particularly in human cancers,[20-24] including urological cancers.[25,26] The roles and mechanisms of circRNAs in different types of human cancers have been largely explored recently. circRNAs inhibit or promote tumor cell growth via regulating the expression of miRs like sponges. In human kidney, the suppression of circHIAT1 is involved in androgen receptor-enhanced migration and invasion of renal clear cell carcinoma by deregulating miR-195-5p/29a-3p/29c-3p expression.[26] In addition, peripheral blood hsa_circ_0124644 significantly elevates in coronary artery disease,[27] and hsa_circRNA_103636 gradually decreases along with the effective treatment in major depressive disorder.[28] These findings suggest that circRNAs play important roles in the pathogenesis of human diseases and might serve as novel diagnostic biomarkers and therapeutic targets.

However, circRNAs have not been reported in any glomerular or tubulointerstitial diseases. We found previously that miR-150 significantly increased in repeated renal biopsies of LN patients with high chronicity index (CI) and promoted renal fibrosis by regulating fibrosis-associated genes.[29] Many other miRs have also been demonstrated to be involved in the pathogenesis of LN.[30-31] So far, the profiling of circRNAs and their roles in LN remain completely unknown in either patients or animal models.

In this study, we aimed to identify circRNA profiling in renal biopsies from LN patients and explore the roles of circRNAs in the pathogenesis of LN. We showed that circHLA-C remarkably increased while miR-150 significantly decreased in the renal biopsies from new-onset LN patients before the treatment of steroid and immunosuppressant. A perfect match seed type of binding sequence was found between circHLA-C and miR-150.

Received 7 August 2017; accepted 14 December 2017;
https://doi.org/10.1016/j.omtn.2017.12.006.

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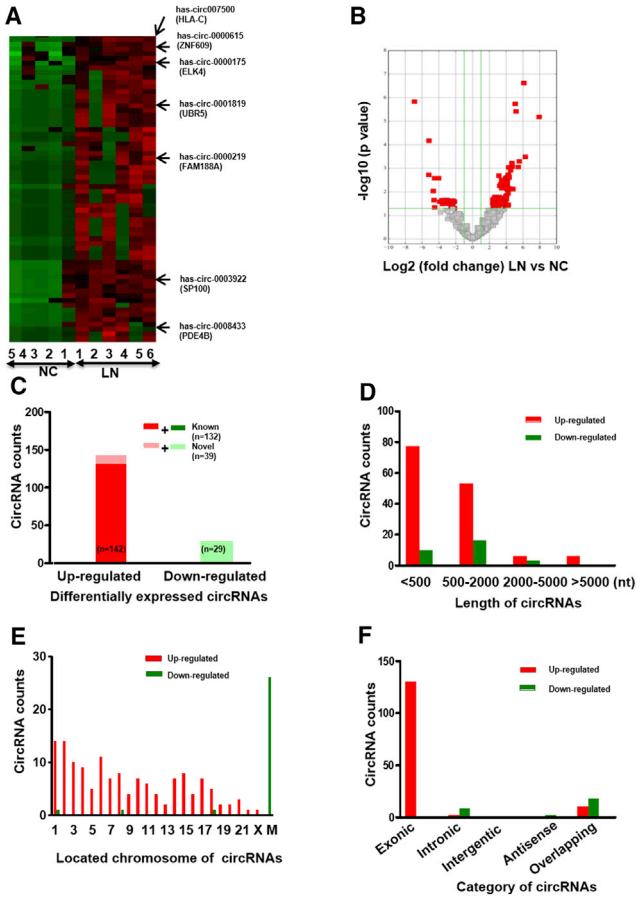

Figure 1. The Profiling and Characteristics of circRNAs in Renal Biopsies from Patients with Lupus Nephritis and Normal Kidney Tissues

(A) Clustered heatmap with each column representing a kidney tissue and each row representing a circular RNA identified by circRNA sequencing. The red indicates upregulated circRNAs, and the green indicates downregulated circRNAs. The seven circRNAs were validated by real-time qPCR (arrows). (B) Volcano plots showed differential expression of circRNAs between lupus nephritis (LN) and normal control kidneys (NC). Vertical line expressed as 2-fold (log2 scaled) up or down changes; the horizontal line represented a p value of 0.05 (−log10 scaled). Red spots indicated the differentially expressed circRNAs with statistical significance. (C) 171 circRNAs showed significant differential expression with over 2-fold change ($p < 0.05$) in LN group compared with NC group. 142 circRNAs were significantly upregulated (red), and 29 circRNAs were significantly downregulated (green). 39 novel circRNAs were identified (light red or green). (D) The distribution of the differentially expressed circRNAs based on the length of nuclear acids. (E) The distribution of differentially expressed circRNAs based on the location on human chromosomes. (F) The counts of differentially expressed circRNAs based on their categories of circle components.

RESULTS

Clinical Characteristics of the Patients

Since the ratio of female to male of LN prevalence is 9:1,[32] six females and one male with biopsy-proven LN class IV (n = 7) were enrolled in this study. With age and gender match, five females and one male with renal tumor (n = 6) were used as control group of normal kidneys. In LN patients, urinary analysis showed proteinuria and hematuria, and serology displayed decrease of complements, increase of immunoglobulins and γ-globulin, the increased score of systemic lupus erythematous activity of diseases indices (SLEDAI, score is 18 to 26), and different severity of renal dysfunction (Table S1). All LN patients presented as class IV pathological feature with typical "full house" immunofluorescent staining. Normal control (NC) subjects showed normal renal function, urinary analysis with negative or trace proteinuria, and normal serum albumin levels except the isolate renal tumor (Table S2). In addition, we further analyzed clinical data in subgroups of six female LN patients. Based on the levels of 24-hr urinary total protein excretion (URTP) greater than 3.5 g/day and serum albumin less than 30 g/L, three patients presented with nephrotic syndrome and three patients displayed nephritic syndrome. Based on the estimated glomerular filtration rate (eGFR) calculated by the chronic kidney disease epidemiology collaboration (CKD-EPI) equation, three patients had renal dysfunction and three patients had normal renal function (47.29 ± 2.61 versus 123.74 ± 13.19 mL/min/1.73 m^2, $p < 0.05$). Higher CI scores (3.33 ± 1.53 versus 0.33 ± 0.58, $p < 0.05$) and increased percentage of the crescentic glomeruli were seen in renal dysfunction subgroup compared with normal renal function group (Table S3).

Profiling and Characteristics of circRNAs in Renal Biopsies of LN Patients

We first analyzed the profiling of circRNAs in kidney tissues of seven LN patients and six normal kidney tissues by RNA deep sequencing. Volcano plots and hierarchical clustering heatmap showed that the circRNA expression levels were clearly distinguished and clustered between LN renal biopsies and NC kidneys (Figure S1). Since females are predominantly susceptible to lupus than males, we focused on analyzing profiling of circRNAs in the kidneys from only female LN patients and NC kidneys in order to remove the confounding factor of gender. Total 18,505 circRNA transcripts were identified in the human kidney tissues, including 11,411 upregulated circRNAs and 7,094 downregulated circRNAs in LN compared with NC. Differentially expressed circRNAs with statistical significance between the two groups were displayed through fold change and p value (fold change ≥ 2.0 and $p < 0.05$). 171 circRNAs were identified to significantly express differentially between the LN group and NC group. 142 circRNAs were significantly upregulated, and 29 circRNAs were remarkably downregulated more than 2-fold in kidney tissues of LN group compared with NC group on cluster heatmap (Figure 1A) and volcano plots (Figure 1B).

Among the 171 differentially expressed circRNAs, 39 circRNAs were first time identified as novel circRNAs, 132 circRNAs were identified previously and listed in the published circRNA database or articles (Figure 1C). The majority of the 171 identified circRNAs had a length with less than 2,000 nucleotides (nt) (Figure 1D). The circRNAs are located on all of human chromosomes, including 22 autosomes and the X chromosome. There was also some downregulated circRNAs distributed on the mitochondria (Figure 1E). According to the sequence structure of nt contained in circRNAs, circRNAs were

Table 1. The Clinical Characteristics of Patients with Lupus Nephritis

Pt	Age (years)	Gender (M/F)	eGFR (mL/min/1.73 m^2)	SLEDAI (score)	Urine		Serum						Renal Biopsy									CRG/TG (%)
					URTP (g/day)	WBC (counts/μL)	RBC (counts/μL)	Alb (g/L)	IgG (g/L)	IgA (g/L)	C3 (g/L)	C4 (g/L)	γ-Glo (g/L)	Fib (score)	IgM (score)	IgA (score)	IgG (score)	C1q (score)	C3 (score)	C4 (score)	AI (score)	CI (score)	
1	52	F	44.9	22	10.6	5.1	5.9	24.3	7.3	1.3	0.5	0.1	16.7	0	1	3	2	3	3	0	13	5	37.5
2	26	F	138.4	26	0.7	79	321.3	20.1	28.3	6.3	0.3	0.2	42.9	0	2	3	2	3	3	3	10	0	27.3
3	35	F	50.1	22	2.7	271.7	354.1	21.5	12.4	1.7	0.4	0.0	24.5	2	3	3	3	3	3	2	10	3	0.0
4	40	F	46.9	18	15.3	530.7	468.1	21	9.3	3.3	0.4	0.2	13.9	2	2	3	3	3	3	2	13	2	46.2
5	40	F	120.1	20	0.2	35.4	2.9	19.5	19.9	2.1	0.1	0.0	37.2	1	1	3	2	1	3	0	7	0	0.0
6	49	F	112.8	21	3.5	190.1	93.7	24.4	12.6	1.8	0.2	0.0	15.4	2	2	3	2	3	3	0	4	1	0.0
7	39	M	125.2	25	10.9	267.9	105.5	23.2	5.5	0.9	0.4	0.1	12.6	1	1	3	3	3	3	2	13	0	27.8

Pt, patient; M/F, male/female; eGFR, estimated glomerular filtration rate based on CKD-EPI formula; SLEDAI, systemic lupus erythematosus disease activity index; URTP, urinary total proteinuria per day; WBC, urinary white blood cells; RBC, urinary red blood cells; Alb, albumin; IgG, immunoglobulin G; IgA, immunoglobulin A; C3, complement 3; C4, complement 4; γ-Glo, serum γ-globulin; Fib, fibrinogen; C1q, complement 1q; AI, activity index; CI, chronicity index; CRG/TG, crescentic glomeruli/total glomeruli.

divided into five categories, including exonic circRNAs composed of the protein coding exons, intronic circRNAs derived from intron lariats, intergenic circRNAs that consist of unannotated regions of the gene, antisense circRNAs transcribed from antisense regions, and sense overlapping circRNAs that originated from both exon and other sequences. In the 171 identified circRNAs, 76.6% (131/171) were exonic circRNAs, 16.4% (28/171) were sense overlapping circRNAs, and 7% (12/171) of circRNAs were other sources (Figure 1F).

In addition, we also analyzed the profiling of circRNAs in six female LN patients subgrouped into nephrotic and nephritic syndrome or renal dysfunction and normal renal function. Cluster heatmap showed that circRNAs expressed significantly differentially in the kidneys between nephrotic and nephritic LN as well as in the kidneys between renal dysfunction and normal renal function (Figure S2).

The circRNA/miR Interaction Network

Based on the magnitude of fold changes and p value of the differentially expressed circRNAs between six female LN patients and five female NC subjects as well as the known functions of circRNAs related to autoimmune, we ranked the top 20 upregulated and top 20 downregulated circRNAs listed for analyzing the interaction network between circRNAs and miRs (Table 1).

The interaction between circRNAs and miRs were theoretically predicted by conserved seed-matching sequence using TargetScan and miRanda analysis. The analysis showed that all of 40 circRNAs contained their respective miR response elements (MREs). The top five miRs regulated by each of the 40 circRNAs were displayed as a network generated by cytoscape software (Figure 2).

Predicted Functions and Pathways of Differentially Expressed circRNAs in LN

The functions of differentially expressed circRNAs were predicted through the host genes of circRNAs by analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). GO enrichment analysis predicted the functional roles of target host genes based on three aspects, including biological processes, cellular components, and molecular functions. Since the downregulated circRNAs were the minority of the differentially expressed circRNAs and were most located on the mitochondria (Figure 1E), we selected 142 significantly upregulated circRNAs as the focus of this study from GO analysis. We found that the regulation of dendritic cell differentiation and the cytoplasmic mRNA processing body assembly were significantly regulated by the 142 overexpressed circRNAs in LN kidneys (Figure 3A). MHC class protein complex and peptide antigen were also significantly regulated by these 142 circRNAs (Figures 3B and 3C). These four predicted functional genes regulated by 142 upregulated circRNAs are well-known genes associated with the pathogenesis of LN.[32]

The KEGG analysis can define the pathways related to the functions of differentially expressed circRNAs' host genes. 23 pathways

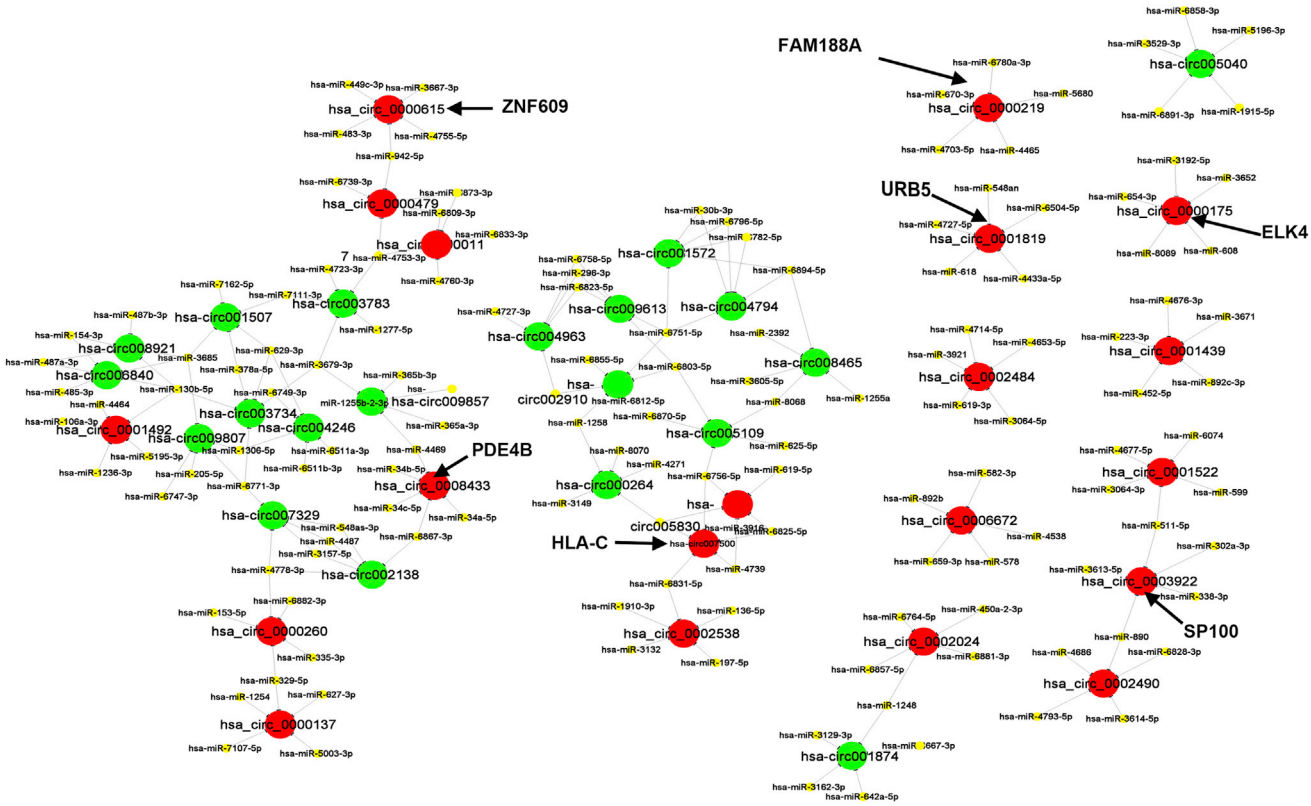

Figure 2. The Interaction Network between circRNAs and miRs

A magnified network showed the top 20 upregulated circRNAs (red) and downregulated circRNAs (green) based on the ranking of fold change, statistical significance, and the involvement of LN disease activity. The top five miRs regulated by each circRNA were expressed (yellow). Seven circRNAs were validated by real-time qPCR (arrows).

related to the functions of 142 upregulated circRNAs in LN were found by the KEGG analysis (data not shown). Among these pathways, hypoxia inducible factor-1 (HIF-1) signaling pathway and neurotrophin signaling pathway are both involved in regulating the expression of NF-κB, which is a well-known important factor in the pathogenesis of LN (Figures 4A and 4B).[33]

Validation of the Selected circRNAs

Since 142 out of 171 differentially expressed circRNAs were upregulated in LN patients, we selected nine upregulated circRNAs (top nine circRNAs in Table 1) and one downregulated circRNA (NUP54) for the validation in the same kidney samples by real-time qPCR based on the ranking order of multiple integrated factors described above and the association with autoimmune diseases. We found that seven out of ten circRNAs showed same change directions and statistical significance as the expression changes on the circRNA sequencing (Figure 5A). These seven validated circRNAs includes circHLA-C, circZNF609, circEKL4, circFAM188A, circUBR5, circPDE4B, and circSP100 were significantly upregulated in LN renal biopsies compared with NC kidneys (Figure 5B). The various bio-features of the seven validated circRNAs, including IDs in a different database (CircBase: http://circbase.org), were summarized (Table S4; Figure 2).

Correlation between circHLA-C and Clinical Disease Activities

We analyzed the correlation between the levels of seven validated circRNAs and traditional clinical indices of disease activity. circHLA-C positively correlated with total proteinuria excretion per day ($R = 0.920$, $p = 0.009$) (Figure 6A), serum creatinine levels ($R = 0.756$, $p = 0.082$) (Figure 6B), percentage of crescentic glomeruli in total glomeruli ($R = 0.929$, $p = 0.007$) (Figure 6C), and renal active index (AI) scores ($R = 0.884$, $p = 0.020$) (Figure 6D). In addition, renal circZNF609, circFAM188A, circELK4, circPDE4B, and circUBR5 significantly correlated with one or two clinical parameters of LN disease activity (data not shown).

Interaction between circHLA-C and miR-150

In this study, renal circHLA-C showed the highest fold change in LN group compared with NC group (4.38 ± 2.00 versus 1.61 ± 0.71, $p < 0.05$) (Figure 7A) among the seven validated circRNAs. In addition, circHLA-C significantly correlated with most clinical parameters of the disease activity (Figure 6). Our previous study reported that miR-150 significantly increased in repeated renal biopsies of LN patients with high CI ($CI \geq 4$).[29] So we selected circHLA-C to investigate its interaction with miR-150. We found that renal miR-150 significantly decreased in LN renal biopsies compared with NC kidneys (0.46 ± 0.19 versus 1.34 ± 0.79, $p < 0.05$) (Figure 7A).

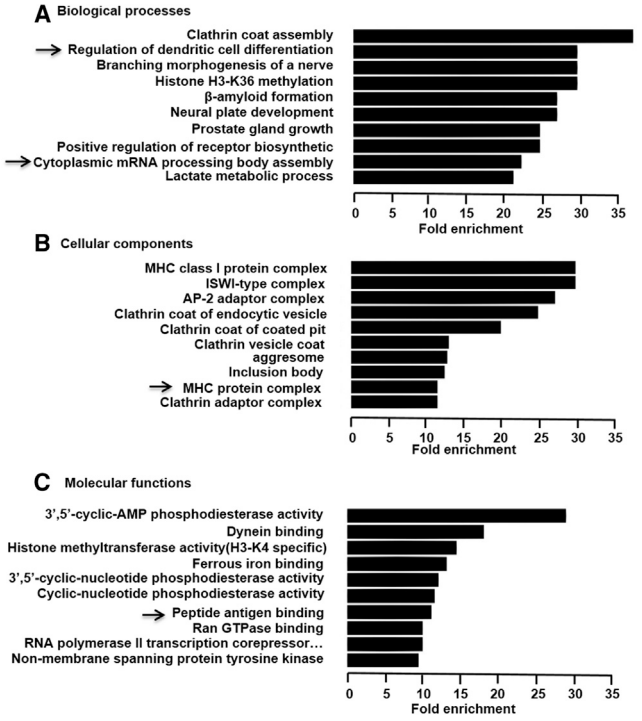

Figure 3. Predicted Functions of the Overexpressed circRNAs in Lupus Nephritis

The top 10 predicted functions of the host gene regulated by 142 overexpressed circRNAs in renal biopsies from patients with lupus nephritis (LN) were obtained with gene oncology (GO) analysis. They were categorized based on (A) biological processes, (B) cellular components, and (C) molecular functions. The fold enrichment expressed that the regulated extent of the predicted functions by the overexpressed circRNAs in LN patients compared with normal control (NC). The arrows indicate the well-known functions involved in the pathogenesis of LN.

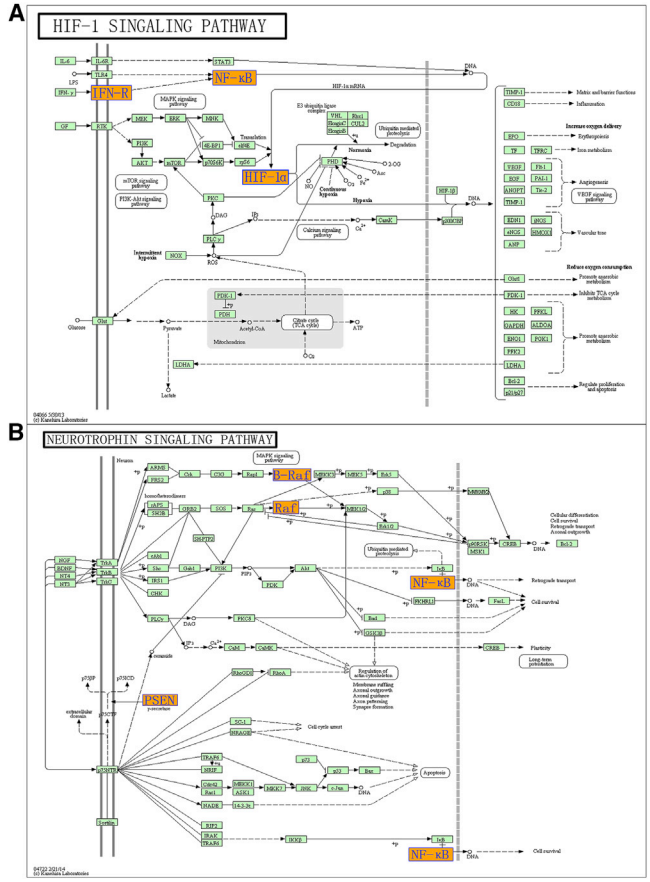

Figure 4. HIF-1 and Neurotrophin Signal Pathways Regulated by the Overexpressed circRNAs in Lupus Nephritis

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed 23 signal pathways relate to the 142 differentially upregulated circRNAs in patients with lupus nephritis. (A) Hypoxia-inducible factor-1 (HIF-1) and (B) neurotrophin signal pathways were 2/23 pathways obtained from KEGG analysis. Both pathways were associated with activation of NF-κB, which is an important factor in the pathogenesis of lupus nephritis.

Renal miR-150 showed a tendency of negative correlation with circHLA-C (Figure 7B).

Then we further analyzed the binding sequence between circHLA-C and miR-150 interaction by TargetScan and miRanda analysis. We found circHLA-C had a perfect match sequence (7-mer-m8 seed type) to bind miR-150 (Figure 7C).

DISCUSSION

We have identified 171 circRNAs with significantly differential expression and validated seven circRNAs with significant upregulation in kidneys in LN class IV patients compared with NC group. circHLA-C positively correlated with LN disease activity. circHLA-C significantly increased and miR-150 decreased in LN patients compared with normal group. In addition, circHLA-C displayed a tendency of negative correlation with miR-150. A perfect match seed type of binding sequence was shown between circHLA-C and miR-150.

In the last decade, over ten thousand different circRNAs were discovered in various organisms in several human diseases.[3] Recent studies demonstrated the expression of circRNAs as a cell-type-specific and tissue-specific manner.[5,8,9] In terms of the expression of circRNAs in the kidney, Wang et al.[26] reported that circHIAT1 expression was lower in patients with clear cell renal carcinomas than adjacent normal tissues, and Cheng and Joe[34] reported renal circRNA profiling in rat model with hypertension. Beside this single renal cancer study, the profiling of circRNAs in human kidney diseases remains unreported. In this study, we identified 171 differentially expressed circRNAs in renal biopsies of LN compared with NC kidney tissues (Figure 1). Seven circRNAs with circHLA-C on the top were significantly increased in kidneys of LN patients (Figure 5). To our knowledge, this is the first time the renal circRNA expressions in human glomerular diseases have been reported. circRNAs showed higher expressions than their corresponding linear transcripts[35] and could carry disease information into biological fluids. These features suggest that circRNAs may serve as potential disease biomarkers.[36] The

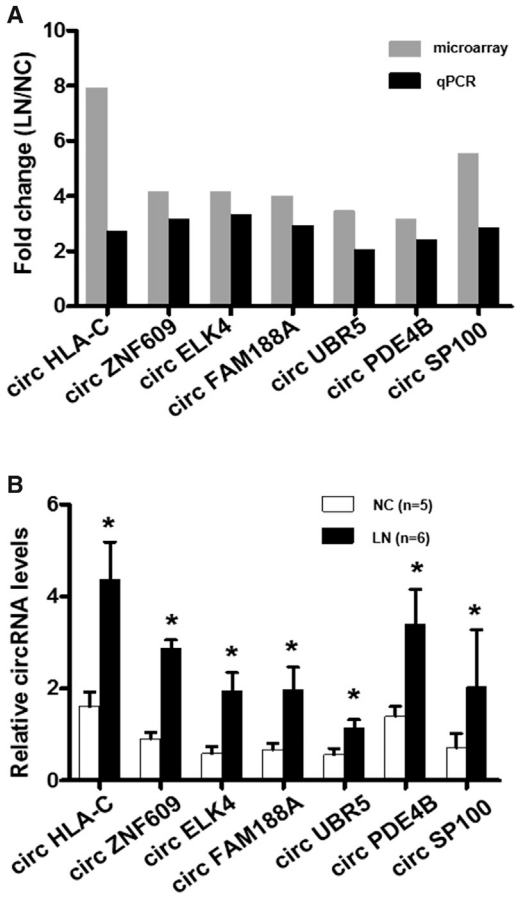

Figure 5. Seven Overexpressed circRNAs in Lupus Nephritis Compared with Normal Control

(A) Seven circRNAs showed same upregulation and statistical significance on real-time qPCR validation as on circRNA sequencing. The upregulated magnitudes of the seven circRNAs were expressed as a fold change of lupus nephritis (LN) to normal control (NC). (B) The relative levels of the seven circRNAs by real-time qPCR analysis in the kidneys from LN compared with NC. The levels of circRNAs from RNA sequencing were expressed as fold-change ratio of LN to NC (log2 transformed) for each circRNA. The renal levels of circRNA from real-time qPCR were expressed as a ratio of average $2^{-\Delta\Delta Ct}$ of each circRNA form LN to NC. Data are expressed as means ± SD (*$p < 0.05$).

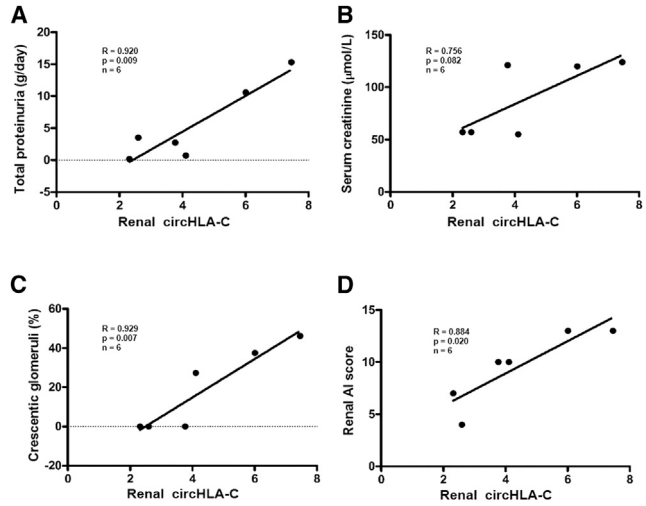

Figure 6. The Correlation between Renal circHLA-C and Clinical Parameters in Lupus Nephritis

(A) circHLA-C positively correlated with total proteinuria excretion per day. (B) A tendency of positive correlation between circHLA-C and serum creatinine. circHLA-C positively correlated with the severity of active kidney tissue damage, including (C) percentage of crescentic glomeruli and (D) score of renal activity index (AI score).

differentially expressed circRNAs from this study may provide a database of circRNAs to study a novel class of biomarkers for LN.

circRNAs have been demonstrated to function as miR sponges or potent competitive endogenous RNA (ceRNA) molecules.[37,38] An interaction network between hundreds of circRNAs and miRs was predicted in the present study (Figure 2). This network provided plenty of valuable information to study circRNAs and their targeted miRs. The majority of the identified circRNAs from our study contained miR response elements, and this suggests that these circRNAs might be involved in pathogenesis of LN by regulating the expression of miRs. In addition, GO enrichment analysis revealed that the upregulated circRNAs were involved in the multiple biological functions such as regulation of dendritic cell (DC) differentiation, cytoplasmic mRNA processing body assembly, MHC protein complex, and peptide antigen binding (Figure 3). Cehlar et al.[39] reported that the increase of Toll-like receptor 7 in DCs is central to the development of LN. Glomerular accumulation of DCs and introduction of DC differentiation were seen at inflammatory sites in kidney tissues of active LN.[40,41] Raj et al.[42] reported that DC gene expression of HLA-D region and antigen-presentation pathways were upregulated in lupus patients. These data suggest that the roles of the overexpressed circRNAs in LN development might be mediated through regulating the above biological functions. On the other hand, KEGG analysis revealed two pathways that related to activation of NF-κB signaling (Figure 4). NF-κB can promote the progression of LN by increasing inflammatory response.[33] This can explain the possible mechanisms of the increased circRNAs in LN class IV.

We also found six out of seven validated circRNAs correlated with different parameters of LN disease activity. circHLA-C was significantly positively correlated with 24-hr urinary protein excretion, percentage of crescentic glomeruli in total glomeruli, and renal activity index. In addition, circHLA-C also showed a tendency of positive correlation with serum creatinine. The significant correlation between circHLA-C and LN disease activities was easily shown in such small numbers of LN patients, we believe that circHLA-C or other circRNAs most likely correlate with more disease activities in larger numbers of LN patients. These data suggest that circHLA-C might be involved in the development of LN and might promote the progress of LN.

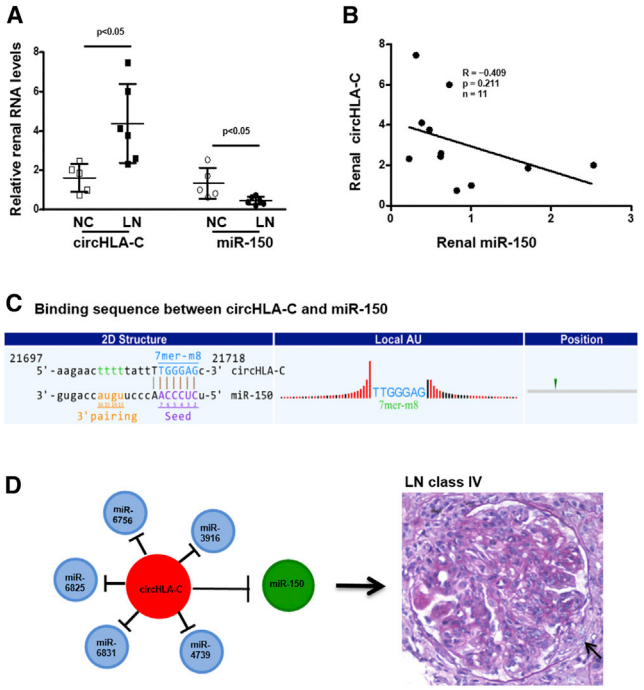

Figure 7. The Interaction between Renal circHLA-C and miR-150 in Lupus Nephritis

(A) The relative expression levels of renal circHLA-C and miR-150 between normal control (NC) and lupus nephritis (LN) by real-time qPCR. Data are expressed as means ± SD. (B) A tendency of negative correlation between renal miR-150 and renal circHLA-C. (C) The interaction between circHLA-C and miR-150 was predicted by TargetScan and miRanda. The "2D Structure" column shows the binding sequence of circHLA-C and miR-150 as a perfect match seed type (7-mer-m8). The "Local AU" column displays 30 nucleotides in the upstream and downstream of the seed sequence. The "Position" column indicates the probable position of miR-150 response element on the linear presentation of circHLA-C. (D) Schematic cartoon of circHLA-C in pathogenesis of LN by sponging miR-150 and other miRs. The histology image was from the renal biopsy report from patient #4 listed in Table S1 (arrow indicated a cellular crescent).

We previously reported that miR-150 significantly increased in repeated renal biopsies of 14 LN patients with high CI (CI $\geq$ 4, n = 14) compared with the LN patients with low CI (CI < 4, n = 11). The renal miR-150 positively correlated with renal CI in the inside of LN patients.[29] In the present study, we found that miR-150 expression decreased in LN renal biopsies compared with NC kidneys (Figure 7A). Only one out of six patients had high CI score (CI = 5) (Table S1), and all patients were new onset of LN and did not receive any treatment of steroid and/or immunosuppressants in current study. Based on our two studies, we speculate that renal miR-150 expression might be different along with the changes of LN disease activities and the status of the treatment. We also found a tendency of negative correlation and one perfect match seed between circHLA-C and miR-150 (Figures 7B and 7C). This suggests that the upregulated circHLA-C might sponge miR-150 in the new onset of LN class IV to promote active kidney damage, including the formation of crescents in the glomeruli and excretion of proteinuria. circHLA-C, a novel circRNA, has not been reported in circRNA database so far. We first-time identified circHLA-C and found it might play an important role in the pathogenesis of LN by sponging miR-150. Of course, the other miRs displayed on the downstream top 5 target miRs of circHLA-C might also participate in the pathogenesis of LN (Figure 7D).

The present study focused on the most common single class IV of LN in six female patients. Although this minimalized the confounder factors due to the complexity of LN classification, it might also miss some circRNA signatures existing in other types of LN. Since the difficulty to obtain more renal biopsies from LN patients, the validation cohort is small. The overexpression of circHLA-C in human renal cells and the inhibition of circHLA-C in LN model animals in future will help on understanding the roles and mechanisms of circRNAs in the pathogenesis of LN and estimate the therapeutic values of circRNAs in LN. The examination of circHLA-C in blood or urine samples from large cohort LN patients may define its clinical values as a novel biomarker.

Our data, for the first time, provided a renal circRNA profiling and an interaction network between circRNAs and miRs in LN. The significant correlation between the overexpressed circHLA-C and LN disease activities indicates that circHLA-C plays an important role in the pathogenesis of LN. The downregulation of renal miR-150 and perfect miR-150 response element on circHLA-C suggest that circHLA-C might participate in the development of LN by sponging miR-150. Further study needs investigation of circHLA-C in larger cohorts of LN patients and mechanisms of circHLA-C in LN *in vitro* and *in vivo* experiments.

MATERIALS AND METHODS

Human Kidney Tissue Samples

Seven LN patients with renal biopsy proven as class IV, including six females and one male, were prospectively enrolled in this study between January and October 2016 from Department of Nephrology at the First Hospital of China Medical University. Six patients with renal tumor as NC group, including five females and one male, were from the Urology Department at the same hospital. We excluded individuals with eGFR lower than 30 mL/min/1.73 m^2, age younger than 18 or older than 60, pregnancy, hepatitis virus infection, hypertension, or diabetes in all human subjects. We also excluded any tumor patients from LN group.

A human subject research protocol was approved in advance by the Institutional Review Boards of the First Hospital of China Medical University. All subjects provided written informed consent prior to research participation.

All renal biopsy tissues from LN patients were obtained before the treatment with steroid and/or immunosuppressant. Kidney tissues as NC were obtained from renal tumor patients at least 5 cm from the edge of renal tumor. The NC tissues were additionally stained with periodic acid-Schiff and were confirmed to be normal

histological morphology under microscopy by a nephrology pathologist. All kidney tissues were stored in −80°C until RNA extraction.

circRNA Profiling Analysis

Total RNA was extracted from the frozen kidney tissues with using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The concentration of each RNA sample was determined by NanoDrop ND-1000 analysis (Agilent, Wilmington, DE, USA). All RNA samples used in this study passed the quality control based on a qualified ratio of OD260 to OD280 (1.8∼2.1).

RNA library preparation and circRNA sequencing were performed by CloudSeq Biotech (Shanghai, China). For each sample, 5 μg of total RNA was incubated for 15 min at 37°C with 3 U/μg of RNase R (Epicenter, Madison, WI, USA) to enrich circRNAs. The Rnase-R treated RNA was then rRNA depleted using the Ribo-Zero Magnetic Gold Kit (Epicenter, Madison, WI, USA). The rRNA-depleted RNA was used to construct the RNA libraries with TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The library quality was evaluated with BioAnalyzer 2100 system (Agilent Technologies, Richardson, TX, USA). The RNA libraries were denatured as single-stranded DNA molecules. The cDNAs were captured on Illumina Flow Cells (Illumina, San Diego, CA, USA), amplified *in situ* as clusters and finally sequenced with 150-bp paired reads on HiSeq 4000 sequencing system (Illumina, San Diego, CA, USA).

To generate the profiling of differentially expressed circRNAs between LN kidneys and NC kidneys, the hierarchical clustering analysis was performed based on the expression levels of all identified circRNAs and the significant difference between LN and control kidneys by Cluster and TreeView software. The predicted functions of the differentially expressed circRNAs between LN and NC were obtained by GO and KEGG analysis. The interaction seed between circHLA-C and miR-150 were predicted by TargetScan and miRanda, and the network of circRNAs and their downstream miRs was generated by cytoscape software (v2.8.0).

Real-Time qPCR of circRNAs and miR-150

Real-time qPCR with SYBER green analysis was used to validate the expression of the selected circRNAs from circRNA sequencing. Total RNAs (0.8 μg) were used for first-strand cDNA synthesis with dNTP Mix (HyTestLtd, Turku, Finland), SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Chino, CA, USA), and RNase Inhibitor (Enzymatics, Green Bay, WI, USA). The first strand cDNA (2 μL) was used for PCR by the ViiA 7 Real-Time PCR System analysis (Applied Biosystems, Foster City, CA, USA). The relative expression levels of circRNAs were expressed as $2^{-\Delta\Delta Ct}$ of each circRNA measurement. Gapdh was used as an endogenous control gene for circRNAs. The relative expression level of the miR-150 was analyzed by the same real-time qPCR method used in validation of the circRNAs. U6 was used as an endogenous control gene for miR-150.

Statistical Analysis

Statistical software SPSS 17.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Quantitative data were expressed as mean ± SD. Differences between two groups were analyzed for statistical significance by t test. Correlation between two variations was analyzed by Pearson's linear correlation analysis. A p value < 0.05 was accepted as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and four tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2017.12.006.

AUTHOR CONTRIBUTIONS

J.L., C.J., and W.K contributed equally to this work, including kidney sample collection, clinical data collection, data analysis, and manuscript writing. H.Z. designed and conducted the whole experiment and finalized the manuscript. G.G. and H.Q. participated in sample and data collection. L.Y., L.W., J.F., and J.P. participated in the design of experiments. W.Q. and Y.C. conducted kidney biopsies from patients with lupus nephritis. X.Z. and Y.Z. collected kidney tissues from patients with renal tumor.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by the National Science Foundation of China (81370835 and 81770698) and the Pandeng Scholar of Education Department of Liaoning Province (2013222).

REFERENCES

1. Almaani, S., Meara, A., and Rovin, B.H. (2017). Update on lupus nephritis. Clin. J. Am. Soc. Nephrol. *12*, 825–835.
2. Tektonidou, M.G., Dasgupta, A., and Ward, M.M. (2016). Risk of end-stage renal disease in patients with lupus nephritis, 1971-2015: a systematic review and bayesian meta-analysis. Arthritis Rheumatol. *68*, 1432–1441.
3. Chen, L.L. (2016). The biogenesis and emerging roles of circular RNAs. Nat. Rev. Mol. Cell Biol. *17*, 205–211.
4. Jeck, W.R., Sorrentino, J.A., Wang, K., Slevin, M.K., Burd, C.E., Liu, J., Marzluff, W.F., and Sharpless, N.E. (2013). Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA *19*, 141–157.
5. Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., Maier, L., Mackowiak, S.D., Gregersen, L.H., Munschauer, M., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. Nature *495*, 333–338.
6. Yang, Y., Fan, X., Mao, M., Song, X., Wu, P., Zhang, Y., Jin, Y., Yang, Y., Chen, L.L., Wang, Y., et al. (2017). Extensive translation of circular RNAs driven by N6-methyladenosine. Cell Res. *27*, 626–641.
7. Legnini, I., Di Timoteo, G., Rossi, F., Morlando, M., Briganti, F., Sthandier, O., Fatica, A., Santini, T., Andronache, A., Wade, M., et al. (2017). Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis. Mol. Cell *66*, 22–37.e9.
8. Salzman, J., Chen, R.E., Olsen, M.N., Wang, P.L., and Brown, P.O. (2013). Cell-type specific features of circular RNA expression. PLoS Genet. *9*, e1003777.
9. Rybak-Wolf, A., Stottmeister, C., Glažar, P., Jens, M., Pino, N., Giusti, S., Hanan, M., Behm, M., Bartok, O., Ashwal-Fluss, R., et al. (2015). Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. Mol. Cell *58*, 870–885.

10. Tan, W.L., Lim, B.T., Anene-Nzelu, C.G., Ackers-Johnson, M., Dashi, A., See, K., Tiang, Z., Lee, D.P., Chua, W.W., Luu, T.D., et al. (2017). A landscape of circular RNA expression in the human heart. Cardiovasc. Res. *113*, 298–309.

11. Geng, H.H., Li, R., Su, Y.M., Xiao, J., Pan, M., Cai, X.X., and Ji, X.P. (2016). The circular RNA Cdr1as promotes myocardial infarction by mediating the regulation of miR-7a on its target genes expression. PLoS ONE *11*, e0151753.

12. Wang, K., Long, B., Liu, F., Wang, J.X., Liu, C.Y., Zhao, B., Zhou, L.Y., Sun, T., Wang, M., Yu, T., et al. (2016). A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. Eur. Heart J. *37*, 2602–2611.

13. Devaux, Y., Creemers, E.E., Boon, R.A., Werfel, S., Thum, T., Engelhardt, S., Dimmeler, S., and Squire, I.; Cardiolinc Network (2017). Circular RNAs in heart failure. Eur. J. Heart Fail. *19*, 701–709.

14. Shao, Y., and Chen, Y. (2016). Roles of circular RNAs in neurologic disease. Front. Mol. Neurosci. *9*, 25.

15. Chen, B.J., Mills, J.D., Takenaka, K., Bliim, N., Halliday, G.M., and Janitz, M. (2016). Characterization of circular RNAs landscape in multiple system atrophy brain. J. Neurochem. *139*, 485–496.

16. Li, T.R., Jia, Y.J., Wang, Q., Shao, X.Q., and Lv, R.J. (2017). Circular RNA: a new star in neurological diseases. Int. J. Neurosci. *127*, 726–734.

17. Cardamone, G., Paraboschi, E.M., Rimoldi, V., Duga, S., Soldà, G., and Asselta, R. (2017). The Characterization of GSDMB splicing and backsplicing profiles identifies novel isoforms and a circular RNA that are dysregulated in multiple sclerosis. Int. J. Mol. Sci. *18*, E576.

18. Liu, Q., Zhang, X., Hu, X., Yuan, L., Cheng, J., Jiang, Y., and Ao, Y. (2017). Emerging roles of circRNA related to the mechanical stress in human cartilage degradation of osteoarthritis. Mol. Ther. Nucleic Acids *7*, 223–230.

19. Zhao, Z., Li, X., Jian, D., Hao, P., Rao, L., and Li, M. (2017). Hsa_circ_0054633 in peripheral blood can be used as a diagnostic biomarker of pre-diabetes and type 2 diabetes mellitus. Acta Diabetol. *54*, 237–245.

20. Dong, Y., He, D., Peng, Z., Peng, W., Shi, W., Wang, J., Li, B., Zhang, C., and Duan, C. (2017). Circular RNAs in cancer: an emerging key player. J. Hematol. Oncol. *10*, 2.

21. Wang, F., Nazarali, A.J., and Ji, S. (2016). Circular RNAs as potential biomarkers for cancer diagnosis and therapy. Am. J. Cancer Res. *6*, 1167–1176.

22. He, J., Xie, Q., Xu, H., Li, J., and Li, Y. (2017). Circular RNAs and cancer. Cancer Lett. *396*, 138–144.

23. Yu, L., Gong, X., Sun, L., Zhou, Q., Lu, B., and Zhu, L. (2016). The circular RNA Cdr1as act as an oncogene in hepatocellular carcinoma through targeting miR-7 expression. PLoS ONE *11*, e0158347.

24. Sand, M., Bechara, F.G., Gambichler, T., Sand, D., Bromba, M., Hahn, S.A., Stockfleth, E., and Hessam, S. (2016). Circular RNA expression in cutaneous squamous cell carcinoma. J. Dermatol. Sci. *83*, 210–218.

25. Zhong, Z., Lv, M., and Chen, J. (2016). Screening differential circular RNA expression profiles reveals the regulatory role of circTCF25-miR-103a-3p/miR-107-CDK6 pathway in bladder carcinoma. Sci. Rep. *6*, 30919.

26. Wang, K., Sun, Y., Tao, W., Fei, X., and Chang, C. (2017). Androgen receptor (AR) promotes clear cell renal cell carcinoma (ccRCC) migration and invasion via altering the circHIAT1/miR-195-5p/29a-3p/29c-3p/CDC42 signals. Cancer Lett. *394*, 1–12.

27. Zhao, Z., Li, X., Gao, C., Jian, D., Hao, P., Rao, L., and Li, M. (2017). Peripheral blood circular RNA hsa_circ_0124644 can be used as a diagnostic biomarker of coronary artery disease. Sci. Rep. *7*, 39918.

28. Cui, X., Niu, W., Kong, L., He, M., Jiang, K., Chen, S., Zhong, A., Li, W., Lu, J., and Zhang, L. (2016). hsa_circRNA_103636: potential novel diagnostic and therapeutic biomarker in major depressive disorder. Biomarkers Med. *10*, 943–952.

29. Zhou, H., Hasni, S.A., Perez, P., Tandon, M., Jang, S.I., Zheng, C., Kopp, J.B., Austin, H., 3rd, Balow, J.E., Alevizos, I., and Illei, G.G. (2013). miR-150 promotes renal fibrosis in lupus nephritis by downregulating SOCS1. J. Am. Soc. Nephrol. *24*, 1073–1087.

30. Dai, Y., Sui, W., Lan, H., Yan, Q., Huang, H., and Huang, Y. (2009). Comprehensive analysis of microRNA expression patterns in renal biopsies of lupus nephritis patients. Rheumatol. Int. *29*, 749–754.

31. Lu, J., Kwan, B.C., Lai, F.M., Tam, L.S., Li, E.K., Chow, K.M., Wang, G., Li, P.K., and Szeto, C.C. (2012). Glomerular and tubulointerstitial miR-638, miR-198 and miR-146a expression in lupus nephritis. Nephrology (Carlton) *17*, 346–351.

32. Zharkova, O., Celhar, T., Cravens, P.D., Satterthwaite, A.B., Fairhurst, A.-M., and Davis, L.S. (2017). Pathways leading to an immunological disease: systemic lupus erythematosus. Rheumatology (Oxford) *56* (*Suppl 1*), i55–i66.

33. Jiang, T., Tian, F., Zheng, H., Whitman, S.A., Lin, Y., Zhang, Z., Zhang, N., and Zhang, D.D. (2014). Nrf2 suppresses lupus nephritis through inhibition of oxidative injury and the NF-κB-mediated inflammatory response. Kidney Int. *85*, 333–343.

34. Cheng, X., and Joe, B. (2017). Circular RNAs in rat models of cardiovascular and renal diseases. Physiol. Genomics *49*, 484–490.

35. Bahn, J.H., Zhang, Q., Li, F., Chan, T.M., Lin, X., Kim, Y., Wong, D.T., and Xiao, X. (2015). The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva. Clin. Chem. *61*, 221–230.

36. Memczak, S., Papavasileiou, P., Peters, O., and Rajewsky, N. (2015). Identification and characterization of circular RNAs as a new class of putative biomarkers in human blood. PLoS ONE *10*, e0141214.

37. Hansen, T.B., Jensen, T.I., Clausen, B.H., Bramsen, J.B., Finsen, B., Damgaard, C.K., and Kjems, J. (2013). Natural RNA circles function as efficient microRNA sponges. Nature *495*, 384–388.

38. Zheng, Q., Bao, C., Guo, W., Li, S., Chen, J., Chen, B., Luo, Y., Lyu, D., Li, Y., Shi, G., et al. (2016). Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. Nat. Commun. *7*, 11215.

39. Celhar, T., Hopkins, R., Thornhill, S.I., De Magalhaes, R., Hwang, S.H., Lee, H.Y., Yasuga, H., Jones, L.A., Casco, J., Lee, B., et al. (2015). RNA sensing by conventional dendritic cells is central to the development of lupus nephritis. Proc. Natl. Acad. Sci. USA *112*, E6195–E6204.

40. Blanco, P., Palucka, A.K., Gill, M., Pascual, V., and Banchereau, J. (2001). Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science *294*, 1540–1543.

41. Tucci, M., Quatraro, C., Lombardi, L., Pellegrino, C., Dammacco, F., and Silvestris, F. (2008). Glomerular accumulation of plasmacytoid dendritic cells in active lupus nephritis: role of interleukin-18. Arthritis Rheum. *58*, 251–262.

42. Raj, P., Rai, E., Song, R., Khan, S., Wakeland, B.E., Viswanathan, K., Arana, C., Liang, C., Zhang, B., Dozmorov, I., et al. (2016). Regulatory polymorphisms modulate the expression of HLA class II molecules and promote autoimmunity. eLife *5*, e12089.